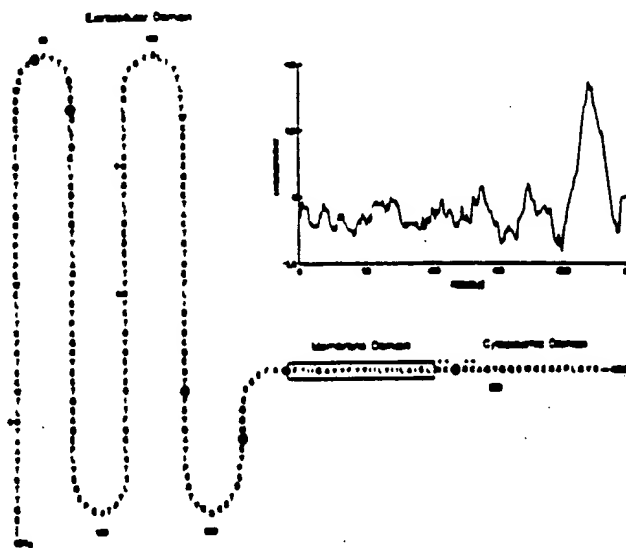




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(54) Title: CLONING AND EXPRESSION OF HUMAN TISSUE FACTOR



(57) Abstract

Recombinant cloning vectors replicable in a suitable host which contain a DNA fragment the sequence of which codes for human tissue factor, a procoagulant protein which triggers the extrinsic pathway of coagulation, are provided. Recombinant bacteriophage λ 10,3 which contains a 2147 bp cDNA insert which codes for tissue factor was obtained by screening a human placental cDNA library cloned into λ t11 for tissue factor coding sequences and purifying the recombinant bacteriophage containing said sequences. The 2147 bp cDNA fragment coding for human tissue factor and the substantially pure human tissue factor encoded therefrom are also provided. Also provided are a recombinant plasmid pKS-2B which contains the 2147 bp cDNA coding for tissue factor, as well as recombinant vectors which in suitable host cells express human tissue factor and soluble human tissue factor. Furthermore, substantially pure human tissue factor and novel soluble tissue factor are provided.

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 88/01915

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁴: C 12 N 15/00; C 07 H 21/04; C 12 Q 1/68; C 12 P 21/02

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System

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Documentation Searched other than Minimum Documentation
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III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Thrombosis and Haemostasis, volume 58, no. 1, January 1987, (Stuttgart, DE), T.S. Edgington et al.: "Molecular cloning of human tissue factor cDNA", page 258 see abstract 941	1-3,5,19- 21,23
X	-- Federation Proceedings, volume 46, no. 6, 1 May 1987, 78th Annual Meeting, Philadelphia, PA, 7-11 June 1987, American Society of Biological Chemists, (Bethesda, Maryland, US), E.M. Scarpati et al.: "Human tissue factor: cDNA cloning, primary structure, and chromosome localiza- tion", page 2242 see abstract 1846 cited in the application	1-3,5,19- 21,23
X	-- Federation Proceedings, volume 46, no. 3, 1 March 1987, 71st Annual Meeting, Washington, DC, 29 March - 2 April 1987, (Bethesda, Maryland US), ./. .	1-3,5,19- 21,23

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IV. CERTIFICATION

Date of the Actual Completion of the International Search

6th September 1988

Date of Mailing of this International Search Report

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International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

E.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	J.H. Morrissey et al.: "Molecular cloning of the cDNA for human tissue factor", page 716 see abstract 2338 cited in the application --	
P,X	Proceedings of the National Academy of Sciences of the USA, volume 84, no. 15, August 1987, (Washington, DC, US), E.K. Spicer et al.: "Isolation of cDNA clones coding for human tissue factor: primary structure of the protein and cDNA", pages 5148-5152 see the whole article cited in the application --	1-3,5-7, 9,11,12, 14-18,19- 21,23
P,X	Biochemistry, volume 26, no. 17, 25 August 1987, American Chemical Society, (Washington, DC, US), E.M. Scarpati et al.: "Human tissue factor: cDNA sequence and chromosome localization of the gene", pages 5234-5238 see the whole article cited in the application --	1-3,5,15, 19-21,23
P,X	Cell, volume 50, no. 1, 3 July 1987, Cell Press, (Cambridge, MA, US), J.H. Morrissey et al.: "Molecular cloning of the cDNA for tissue factor, the cellular receptor for the initiation of the coagulation protease cascade", pages 129-135 see the whole article cited in the application --	1-3,5,15, 19-21,23
P,X	Thrombosis Research, volume 48, no. 1, October 1987, Pergamon Journals Ltd, (New York, US), K.L. Fisher et al.: "Cloning and expression of human tissue factor cDNA", pages 89-99 see the whole article --	1-5,15, 19-23
A	Proc. Natl. Acad. Sci. USA, volume 83, January 1986, (Washington, DC, US), A. Guha et al.: "Affinity purification of human tissue factor: interaction of factor VII and tissue factor in detergent micelles", pages 299-302 see abstract; page 301, column 1, ...	15

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
E	lines 26-54 -- EP, A, 0278776 (GENENTECH) 17 August 1988 see claims -----	1-5, 15, 19- 23, 28-31, 33-35, 37- 38

US 8801915
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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

Description

Cloning and Expression of Human Tissue Factor

Specification

Background of the Invention

5 The present invention relates to novel recombinant vectors replicable in a suitable host which contain cloned DNA sequences coding for human tissue factor. The invention also relates to the DNA coding for human tissue factor and to the sub-
10 stantially pure human tissue factor and functional portions thereof encoded therefrom. The invention also provides for transformant hosts containing expression vectors capable of expressing human tissue factor and a novel soluble form of human tissue
15 factor.

 The coagulation (clotting) system in man and animals is a major contributor to the maintenance of hemostasis and also to thrombus (blood clot) formation. Coagulation is essentially a cascade in
20 which each clotting factor, which is normally present in the blood and other tissues as an inactive enzyme precursor, i.e., zymogen, is in sequence activated into a proteolytic enzyme that selectively attacks the
25 next zymogen in the clotting sequence, thereby converting it into an active enzyme. Amplification occurs at each step so that a small initial stimulus ultimately results in a significant amount of fibrin clot; which is the final product of the clotting process.

30 The clotting cascade begins as two separate pathways that ultimately converge. One pathway is "intrinsic" to the blood and the other one is termed "extrinsic" because it is triggered by clotting factors not normally present in blood. It is believed

that the intrinsic pathway plays a major role in hemostasis following injury. The extrinsic pathway can become activated in a variety of pathologic situations, e.g., diffuse endothelial damage, advanced
5 cancer, endotoxemia, and pregnancy complications.

There is now considerable evidence that coagulation is started in the body when factor VII, a vitamin K-dependent plasma clotting factor protein and tissue factor, a cell-bound protein not normally
10 associated with blood cells, interact. (See e.g. Nemerson, Blood 71:1-8, 1988 for a review). This interaction results in an activated complex which has enzymatic activity and initiates clotting by converting two other proteins, i.e., factor X and
15 factor IX, to their active, enzymatic forms, factor X_a and factor IX_a, respectively. (In accord with common practice, the zymogen precursor forms of the active blood clotting factors are denoted by a Roman numeral, and the active forms are indicated by a subscripted
20 "a", e.g. factor X for zymogen and factor X_a for activated factor.)

Tissue factor is a procoagulant protein present on the surface of virtually all cells not normally in direct contact with blood. However, tissue factor is
25 inducible in cultured endothelial cells and monocytes upon stimulation with various pharmacologic mediators, e.g. tumor necrosis factor, interleukin-1, and endotoxin. The extrinsic coagulation pathway is triggered by tissue factor which complexes with and
30 activates factor VII, a vitamin-K dependent serine protease zymogen. The activation of factor VII by tissue factor, which is a blood clotting enzyme cofactor, occurs in the presence of calcium and results from a conformational change in factor VII.
35 See, e.g., Nemerson et al., in Progress in Hemostasis and Thrombosis, Spaet, T.H. edit., Grune & Stratton, New York, vol. 6, pp. 237-261, 1982; Carson, Prog. Clin. Pathol. 9:1-14, 1984. The precise

conformational change in factor VII which causes it to become activated and initiate clotting, however, has not yet been determined.

Tissue factor and factor VII are extremely important components of the clotting cascade. The severe bleeding frequently seen in individuals who are markedly deficient in factor VII demonstrates the physiologic significance of the extrinsic pathway of blood clotting. In contrast, individuals deficient in proteins involved in the early steps of the intrinsic pathway of coagulation, i.e., high molecular weight kininogen, prekallikrein, and factor XII, are asymptomatic.

Tissue factor, which is a cell membrane-bound glycoprotein associated with phospholipids, is not normally present in the circulation. When blood vessels are disrupted, however, factor VII, which is a plasma coagulation factor, can complex with tissue factor, thereby forming a catalytically-active species which activates both factor IX (plasma thromboplastin component), a component of the intrinsic pathway, to form factor IX_a and factor X (Stuart factor), which is involved in both the extrinsic and intrinsic pathways of coagulation, to yield factor X_a. Tissue factor is thus an important component of the human clotting pathways. Human tissue factor has important use as a diagnostic reagent to monitor and study clotting disorders and potential use in anticoagulation. Chemical and biological characterization of human tissue factor is thus clearly important to an understanding of coagulation in humans.

Although many aspects of the coagulation cascade have been elucidated, the initiation of clotting in vivo is still not fully understood. Specifically, the role of tissue factor has not been thoroughly investigated, since its role in both the intrinsic and extrinsic pathways of coagulation has only recently been recognized. See, e.g. Nemerson et al., Prog.

Hemostasis & Thrombosis 6:237-261, 1982 and Nemerson, Blood 71:1-8, 1988. In addition, efforts to elucidate the mechanism of tissue factor activity in vitro have been hampered by difficulty in obtaining adequate
5 amounts of pure protein for study.

Tissue factor has been purified to homogeneity from bovine brain in order to facilitate a detailed kinetic analysis of the reactions catalyzed by factor VII and tissue factor. Bach et al., J. Biol. Chem.
10 256:8324-8331, 1981. Classical purification techniques used to isolate the protein, such as immunoaffinity chromatography, even with the use of monoclonal antibodies to bovine tissue factor, are laborious and yield inadequate amounts of protein for
15 detailed experiments and clinical use.

Likewise, small amounts of human tissue factor have been purified from human brains obtained from autopsy material and from term placentas. Broze et al., J. Biol. Chem. 260:10917-10920, 1985; Guha et al., Proc. Natl. Acad. Sci. 83:299-302, 1986. The
20 purified human tissue factor appeared to be a single polypeptide chain which had a molecular weight of 46,000 when analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE)
25 under both reducing and non-reducing conditions. The human protein, like bovine brain tissue factor, is resistant to cleavage by both trypsin and chymotrypsin. Immunologic studies have shown, however, that although bovine and human tissue factors
30 function similarly, there is little cross-reactivity between the proteins from the two species.

Cloning the DNA sequences coding for the entire human tissue factor apoprotein would allow a determination of both the genetic and protein sequence
35 for this biologically important protein. It is known that the gene coding for human tissue factor is located on chromosome 1. In addition to providing information about the DNA sequence coding for tissue

factor and the amino acid sequence of the protein encoded therefrom, important information about the structure of the chromosomal gene can be obtained once the DNA coding for tissue factor is obtained. In addition, expression of the cloned human tissue factor gene in a suitable host would provide a ready source of tissue factor for clinical and diagnostic use as well as for experimental study. The DNA sequences encoding the proteins involved in tissue factor-initiated clotting (i.e., the extrinsic coagulation pathway), with the exception of tissue factor itself, have already been cloned and sequenced.

Recent reports have described the cloning of cDNAs coding for portions of human tissue factor. Morrissey et al., 1987, Fed. Proc. 46:716 (Abstr.); Scarpati et al., 1987, Fed. Proc. 46:2242 (Abstr.). Cloned DNA fragments coding for the entire tissue factor protein were not obtained in these studies. However, after the filing date of U.S. Patent Application Serial No. 062,166 which disclosed the cloning and sequence of the cDNA clone coding for the entire human tissue factor apoprotein, several publications reported the isolation and sequence of cDNA clones coding for human tissue factor. See, e.g. Morrissey et al., Cell 50:129-135, 1987; Spicer et al., Proc. Nat. Acad. Sci. 84:5148-5152, 1987; Scarpati et al., Biochemistry 26:5234-5238 1987; Fischer et al., Blood 48:89-99, 1987.

The present invention provides a complete, single cloned cDNA, the sequence of which codes for the entire human tissue factor apoprotein, the tissue factor apoprotein or a functional portion thereof encoded by the cloned cDNA and recombinant vectors containing the cloned cDNA which code for and can be induced to express the entire human tissue factor apoprotein, truncated soluble human tissue factor protein and functional portions thereof in suitable hosts.

Summary of the Invention

In accordance with the present invention replicable recombinant vectors are described which contain a cloned DNA, the sequence of which codes for the entire human tissue factor apoprotein and a functional portions thereof and also provide for the production of expression vectors which in suitable host cells express functional human tissue factor apoprotein and soluble tissue factor. The recombinant vectors were derived by screening recombinant cloning vectors containing a human placental cDNA library for identifiable cDNA sequences coding for human tissue factor and isolating recombinant vectors containing cDNA coding for the entire human tissue factor apoprotein.

Suitable recombinant cloning vectors in which a cloned placental cDNA library can be made include those which replicate in prokaryotic hosts, such as bacteria, or those which replicate in eukaryotic hosts such as yeast, insect cells and animal or human cells. Suitable prokaryotic vectors include, but are not limited to, plasmids, cosmids and bacteriophage. Suitable eukaryotic vectors include inter alia vaccinia virus, bovine papilloma virus, simian virus (SV₄₀) and baculovirus. It will be readily apparent to those skilled in the art that a wide variety of cloning vectors and hosts can be used in the practice of the invention.

The invention further provides a 2147 base pair (bp) cDNA which codes for the entire human tissue factor apoprotein, the sequence of such cDNA, and the human tissue factor protein and functional portions thereof encoded by the cDNA. The cDNA was identified and isolated from a cloned human placental cDNA library. The mature tissue factor apoprotein, a single polypeptide chain of 263 amino acids, is coded for by an open reading frame (ORF) of the cDNA fragment encompassing nucleotides 112 to 997. The

actual translation product of the mRNA corresponding to the open reading frame is a preprotein of 295 amino acids having a leader sequence or signal peptide of 32 amino which is cleaved postranslationally to form the mature human tissue factor apoprotein.

Furthermore, a 1.25 kb fragment of the 2147 bp cDNA has been isolated which includes nucleotides 90 through 1340 as provided in Formula I. This fragment, which contains the entire open reading frame and 5'- and 3'- flanking sequences was used to localize the tissue factor gene to a 9.5 kb DNA fragment from restriction enzyme digested total human genomic DNA (placental) and to produce recombinant expression vectors which in suitable hosts express the human tissue factor apoprotein and functional portions thereof, including soluble human tissue factor.

The invention further provides recombinant expression vectors which in a suitable transformant host provide for expression of the functional human tissue factor. Suitable expression vectors include those which replicate and express a desired protein in prokaryotic hosts, such as bacteria, or those which replicate in eukaryotic hosts such as yeast, insect cells and animal or human cells. Suitable prokaryotic vectors include, but are not limited to, plasmids, cosmids and bacteriophage. Suitable eukaryotic vectors include inter alia vaccinia virus, bovine papilloma virus, simian virus₄₀ (SV₄₀) and baculovirus. It will be readily apparent to those skilled in the art that a wide variety of cloning vectors and hosts can be used in the practice of the invention.

Of particular importance, such vectors have also allowed for production of a truncated soluble form of the tissue factor protein which is missing the carboxy-terminal hydrophobic membrane spanning-portion of the protein. In particular, the vectors provide for expression of a soluble active tissue factor

comprising the extracellular domain or the approximately N-terminal 219/220 amino acids of the mature human tissue factor apoprotein. Such truncated soluble human tissue factor proteins and functional portions thereof, i.e., peptides derived from the extracellular domain of human tissue factor are especially useful as diagnostic reagents and anticoagulant agents.

Brief Description of the Drawings

10 The present invention is now described with reference to the detailed description, Examples and figures in which

Fig. 1 is a representation of the amino acid sequence of the mature human tissue factor apoprotein and hydropathy plot thereof.

Fig. 2 provides the nucleotide sequence of human tissue factor-specific oligonucleotide probes used to screen a cloned placental cDNA library.

Fig. 3 is a schematic representation of the 2147 bp cDNA which codes for human tissue factor apoprotein and the strategy used to obtain the nucleotide sequence thereof.

Fig. 4 shows the construction of recombinant plasmid pKS-2B and recombinant phage M13/LB2TF.

Fig. 5 is a Southern blot showing hybridization of a portion of the 2147 bp cDNA coding for tissue factor to human placental genomic DNA.

Fig. 6A shows the construction of recombinant plasmid pLB4TF.

Fig. 6B shows the construction of M13/TL131P.

Fig. 6C provides the sequence of oligonucleotides TFAD and G8PST used for site specific mutagenesis.

Fig. 7 shows the construction of the human tissue factor expression plasmid pTL8FQ.

Fig. 8 is a Western blot of human tissue factor produced by E. coli 71-18/pTL8FQ transformants.

Fig. 9 depicts the pMAM/TF shuttle vector for expressing human tissue factor in mammalian cells.

Fig. 10 is a schematic representation of the construction of plasmid pLB5TF.

5 Fig. 11 is a schematic representation of the construction of the soluble tissue factor expression vector pLB6TF.

Fig. 12A is a Western blot of soluble human tissue factor produced by E. coli 71-18/pLB6TF
10 transformants.

Fig. 12B is a Western blot of soluble human tissue factor purified by immunoaffinity chromatography on a monoclonal anti-human tissue factor immunoadsorbent column.

15 Description of the Invention

The present invention provides for replicable recombinant vectors containing a cloned cDNA insert, the sequence of which codes for and, in a suitable host, express the entire human tissue factor
20 apoprotein or functional portions thereof. The recombinant vectors have been obtained by screening for DNA sequences coding for human tissue factor in a cloned human placental cDNA library which had been inserted into a cloning vector replicable in a
25 suitable host. Preferred vectors for cloning the human cDNA library include plasmids, bacteriophage and other vectors replicable in bacteria, and vectors replicable in eukaryotic hosts such as yeast, insect cells and animal or human cells. Prokaryotic vectors
30 include inter alia plasmids, cosmids and bacteriophage. Eukaryotic vectors include inter alia vaccinia virus, bovine papilloma virus, SV₄₀, yeast vectors and baculovirus. A preferred cloning vector is bacteriophage λ gt11, an expression vector described by
35 Young and Davis, Science 222:778-782, 1983. A preferred host organism is E. coli strain K1088, a known host for λ gt11.

The 2147 bp DNA fragment encoding the entire human tissue factor apoprotein was isolated from a human placental cDNA library cloned into λ gt11. Human tissue factor coding sequences were identified in the
5 cloned placental cDNA library by screening recombinant bacteriophages whose DNA hybridized to specific oligonucleotide hybridization probes. The DNA sequences of the hybridization probes correspond to and code for amino acid sequences of short peptide
10 fragments from (i) the amino terminal, (ii) the carboxy terminal and (iii) an internal portion of the tissue factor polypeptide chain.

The present invention, in particular, provides a recombinant bacteriophage λ 10,3 which was derived from
15 the placental cDNA library cloned in phage λ gt11 and contains the cloned 2147 bp cDNA insert which codes for the entire human tissue factor apoprotein obtained from the placental cDNA library. Also encompassed by the invention is a second bacteriophage, λ 3,4 which
20 was also derived from λ gt11 and contains tissue factor coding sequences. Phage λ 3,4 contains a cloned 1616 bp cDNA insert from the human placental bp cDNA library, the nucleotide sequence of which is identical to the 3' portion of the 2147 bp DNA insert of phage
25 λ 10,3 and codes for the carboxy-terminal portion of the tissue factor apoprotein.

The invention also provides for the cloned 2147 bp cDNA coding for the entire human tissue factor apoprotein, the sequence of which is provided in
30 Formula I. The sequence of the DNA is characterized by a single open reading frame extending from an ATG initiation codon at nucleotides 112-114 to a TAA termination codon at nucleotides 997-999 which encodes a single polypeptide chain of 295 amino acids, which
35 is the tissue factor preprotein containing a leader sequence of 32 amino acids. The mature tissue factor apoprotein is a single polypeptide of 263 amino acids whose amino terminal sequence is Ser-Gly-Thr-Thr-Asn.

The preprotein whose sequence is also provided in Formula I (beginning at residue -32) is postranslationally converted to the mature tissue factor apoprotein whose sequence begins at the amino acid residue marked +1 as shown in Formula I. The molecular weight of the mature tissue factor apoprotein (without carbohydrate) was calculated to be approximately 29,600.

The invention also provides for a recombinant plasmid, pKS-2B, which was derived from the E. coli cloning vector pUC19 by inserting an approximately 4.15 kb DNA fragment obtained from λ 10,3 which comprises the 2147 cDNA insert coding for tissue factor plus about 1000 bp of λ DNA flanking each end of the tissue factor cDNA insert into the lacZ' gene of pUC19. pKS-2B has been used to transform E. coli strain 71-18 which is a known host for pUC19. E. coli 71-18/pKS-2B transformants are a preferred source of the 2147 bp cDNA fragment coding for human tissue factor.

The availability of the cloned 2147 bp cDNA coding for tissue factor has allowed characterization of the human genomic gene which codes for the protein. A 1.25 kb DNA fragment encompassing nucleotides 90-1340 shown in Formula I which comprises the open reading frame and some 5'- and 3'- flanking sequences has been obtained by restriction enzyme digest of plasmid pKS-2B. The 1.25 kb fragment hybridized to a 9.5 kb DNA fragment of digested human placental genomic DNA. This 9.5 kb genomic DNA fragment is believed to contain at least 3 introns within the sequence coding for tissue factor. Although the tissue factor gene has been mapped to human chromosome 1, the gene has not heretofore been localized, to a specific genomic DNA fragment. The 1.25kb fragment has also been used to construct expression vectors for production of the entire human

tissue factor apoprotein and soluble human tissue factor in suitable host cells.

Based on the amino acid sequence of the entire human tissue factor apoprotein predicted from the sequence of the cDNA insert of λ 10,3 encoding the protein and confirmed by amino acid sequence analysis of about 70% of the protein, a domain structure is proposed for this integral membrane bound protein. A protein domain can be defined as an independently folded functional region of a protein. Each of the domains of the human tissue factor apoprotein provided by the present invention has unique structural and functional features: (1) a signal peptide or leader sequence region of 32 amino acids which is post translationally removed upon conversion of the preprotein to mature active tissue factor; (2) an extracellular, generally hydrophilic, N-glycosylated domain which comprises the approximately amino terminal 219 amino acids; (3) an approximately 23 amino acid stretch of mainly hydrophobic amino acids comprising approximately amino acids 220 to 242 which is believed to be that portion of the protein which spans the cellular membrane; and (4) the carboxy terminal approximately 21 amino acids comprising approximately amino acid residues 243 to 263 which is believed to be the cytoplasmic domain on the inside of the cell.

Fig. 1 represents what is believed to be the domain structure of the substantially pure mature human tissue factor apoprotein of the invention (from which the 32 amino acid signal peptide is removed), the amino acid sequence of which is provided in Formula I. The several salient features of the proposed domain structure (1-4) of tissue factor can be seen with reference to Fig. 1.

Four potential N-linked carbohydrate attachment sites (Asn-X-Ser/Thr) are found in the molecule. One of these sites occurs in the cytoplasmic domain at the

carboxy terminus and therefore is probably not glycosylated. Of the three sites in the extracellular domain, each of which is marked by a

5 (A) in Fig. 1., two have been identified as bearing carbohydrate by amino acid sequence analysis. As discussed in Example 1 no PTH amino acid could be identified at amino acids 11 and 137 (Formula I) which were predicted from the cDNA sequence to be
10 asparagine. Each of these Asn residues occupies the beginning of a carbohydrate attachment site. In general, carbohydrates are only found on the extracellular protein of membrane-bound glycoproteins like tissue factor, so it would be expected that the
15 carbohydrate would only be found on the extracellular domain.

It is also apparent with reference to Fig. 1 and Formula I that human tissue factor contains a total of five half-cystine residues (circled in Fig. 1), four
20 of which occur within the extracellular domain and are probably disulfide linked. The presence of disulfide bridges had been inferred previously by an observation by Bach et al., J. Biol. Chem. 256:8324-8331, 1981, for bovine tissue factor that reduction with 2-
25 mercaptoethanol in the presence of SDS resulted in loss of tissue factor procoagulant activity, whereas treatment with SDS alone did not. It is assumed that Cys at position 245 of human tissue factor is not involved in intramolecular disulfide bond formation
30 since it would be expected to be segregated on the cytoplasmic domain. Further confirmation that Cys²⁴⁵ of human tissue factor is not involved in intramolecular disulfide bond formation was provided by the observation that CNBr cleavage at the single
35 methionine residue, Met²¹⁰, liberated a carboxy-terminal peptide from purified tissue factor without disulfide bond reduction. Cys²⁴⁵ may form intermolecular disulfide linkages, perhaps modulating tissue factor self-association, as described by Bach

et al., Biochemistry 25:4007-4020, 1986, or by interaction with other substances such as proteins or fatty acids during purification.

Also shown in Fig. 1 is a hydropathy plot determined by the method of Kyte and Doolittle, J. Molec. Biol. 157:105-132, 1982, which graphically depicts the hydrophobic character of the membrane domain of tissue factor. Each value was calculated as the average hydropathic index of a sequence of 21 amino acids and plotted to the middle residue of each sequence. The consecutive stretch of 23 non-polar amino acids at the carboxy terminal region of the protein is characteristic of the domains of integral membrane proteins which span the lipid bilayer of the plasma membrane of cells. In addition, four positively charged amino acids (marked as + in Fig. 1) are observed immediately adjacent to the carboxy side of the hydrophobic region, which is a characteristic feature seen at the interface between membrane and cytoplasmic domains of many integral membrane proteins. (See, e.g., Sabatini et al., J. Cell. Biol. 92:1-21, 1982.) Furthermore, the presumed cytoplasmic domain of tissue factor has features common to both the low density lipoprotein receptor and thrombomodulin in that a comparison of this region in all three proteins indicates that the cytoplasmic domain is short and contains a single Cys residue. (See, e.g., Jackman et al., Proc. Natl. Acad. Sci. 83:8834-8838, 1986 and Yamamoto et al., Cell 39:27-38, 1984).

The defined sequence of the substantially pure human tissue factor as provided in Formula I and its apparent domain structure has allowed the production of soluble human tissue factor which comprises the extracellular domain of the protein or portions thereof which is exportable from transformed hosts containing expression vectors which express soluble tissue factor. The sequence also allows for the production of functional portions of human tissue

factor, i.e., peptides derived from human tissue factor, which can be used as diagnostic reagents and to inhibit the binding of Factor VII to tissue factor. Because intact tissue factor is a membrane-bound hydrophobic protein, in general it is not readily secreted from transformed hosts containing vectors for expression of the entire human tissue factor. The availability of large amounts of the soluble form of tissue factor, however, allows for production of the intact protein. The membrane spanning and cytoplasmic domains of human tissue factor can be produced by suitable techniques, e.g. solid phase peptide synthesis. The intact human tissue factor apoprotein is reconstitutible by covalently attaching the soluble human tissue factor produced by recombinant DNA techniques or other methods with the membrane/cytoplasmic domain(s) produced by peptide synthetic techniques through protein synthetic methods such as fragment condensation or protein semi-synthesis techniques.

The soluble tissue factor of this invention is lacking the hydrophobic membrane-spanning and cytoplasmic domains. Surprisingly, such soluble tissue factor, which has not been previously produced, has some minimal procoagulant activity as measured by the activation of Factor VII and the subsequent cleavage of Factors IX and X in standard two stage clotting assays. The soluble tissue factor, which efficiently binds to Factor VII, also has use as an inhibitor of the procoagulant activity of native human tissue factor. Soluble tissue factor, thus, has great potential importance as a diagnostic reagent as well as a potential anticoagulant agent for clinical use.

The cloned 2147 bp cDNA coding for human tissue factor and plasmid pKS-2B have allowed the construction of recombinant expression vectors which in transformed hosts produce human tissue factor apoprotein and soluble human tissue factor for

clinical and diagnostic use, as well as experimental studies. Such expression vectors include those which replicate and express tissue factor in prokaryotic hosts, such as bacteria, or those which replicate in
5 eukaryotic hosts such as yeast, insect cells and animal or human cells. Suitable prokaryotic vectors include, but are not limited to, plasmids, cosmids and bacteriophage. Suitable eukaryotic vectors include inter alia vaccinia virus, bovine papilloma virus,
10 simian virus₄₀ (SV₄₀), yeast vectors, and baculovirus. It will be readily apparent to those skilled in the art that a wide variety of cloning vectors and hosts can be used in the practice of the invention.

In particular, soluble tissue factor encompassing
15 amino acids approximately 1-219/220, i.e. the extracellular domain of the mature apoprotein, has been prepared by DNA cloning techniques. The DNA coding for soluble active tissue factor was prepared by inserting a stop codon in the tissue factor gene
20 immediately downstream from the nucleotides coding for amino acids 219/220 of the tissue factor protein sequence provided in Formula I. This resulted in deletion of the hydrophobic membrane spanning region (domain 3) as well as the cytoplasmic tail (domain 4),
25 of human tissue factor. (See Fig. 1).

Alternatively, soluble human tissue factor comprising the extracellular domain, with or without the signal peptide, may be generated by other techniques including site specific mutagenesis to
30 produce a truncated soluble protein or by cleavage of the mature protein (without the signal peptide) encoded by the 2147 bp cDNA insert in λ 10,11 with CNBr which cleaves the protein at the unique Met residue at position 210 of the mature protein. Such soluble
35 tissue factor proteins have use as anticoagulants and as diagnostics for clotting disorders. Furthermore, functional portions of the soluble proteins corresponding to peptides derived from the soluble

protein can also be used as anticoagulants and as diagnostic reagents.

Intact human tissue factor apoprotein, having procoagulant activity, may be reconstituted from the soluble tissue factor by addition thereto of amino acids thereto which comprise the hydrophobic and cytoplasmic domains of tissue factor. The hydrophobic and cytoplasmic domains may be added to the soluble tissue factor protein by known protein chemistry techniques, such as fragment condensation and protein semi-synthesis.

The invention also provides for those portions of the human tissue factor and the DNA sequences encoding each portion of the protein which have the structural and functional characteristics of the domains (1)-(4). The invention further provides for the substantially pure soluble tissue factor which has not been previously produced.

E. coli strain 71-18 carrying plasmid pKS-2B has been deposited with the American Type Culture Collection and is assigned Accession No. 67426.

The following examples are provided to illustrate the invention and are not intended to limit the same.

Example 1

25 Amino Acid Sequence Analysis of Purified Human Placental Tissue Factor

Approximately 67% of the amino acid sequence of human placental tissue factor was obtained by protein sequencing techniques prior to obtaining the cloned cDNA coding for tissue factor. A knowledge of the amino acid sequence allowed for the design of the specific oligonucleotide hybridization probes described in Example 2 which were used to screen for DNA sequences which coded for tissue factor in the cloned placental cDNA library. The protein sequencing was performed as follows:

A monoclonal IgG, antibody, HTF1-7B8, against human brain tissue factor that had been purified using

factor VII affinity columns as described by Guha et al., 1986, Proc. Natl. Acad. Sci. 83:299-302, was prepared by Carson et al., Blood 70:490-493, 1987. This antibody, which was obtained from Dr. Steven D. Carson of the University of Colorado Health Sciences Center and Dr. Ronald Bach of the Mount Sinai School of Medicine, was used to prepare an immunoabsorbent column for immunoaffinity isolation of human tissue factor. Tissue factor was extracted from human brain or placental tissue acetone powders with the detergent octylphenoxy polyethoxy (10) ethanol (Triton® X-100) and then adsorbed onto the immunoaffinity column. Following washing with Triton® X-100-containing buffers at pH 7.5, the protein was eluted from the column at pH 2.5. It was then concentrated and further purified on an Ultragel AcA 34 column in Triton® X-100, which separated tissue factor from co-eluting minor contaminants to yield a substantially homogeneous protein preparation. The purity of each preparation was assessed by SDS-PAGE (Laemmli, Nature 227:680-685, 1970). The purification was monitored by measuring the tissue factor procoagulant activity in the preparation by a standard two-stage coagulation assay following reconstitution of the detergent-solubilized protein into phospholipid vesicles as described by Bach et al., J. Biol. Chem. 256:8324-8331, 1981.

The amino acid composition and protein concentration of each tissue factor preparation was determined by amino acid analysis: 10 µg of human tissue factor apoprotein in 0.1M NaCl, 0.05M Tris (pH 7.5), and 0.1% Triton® X-100 were precipitated by the addition of 10% trichloroacetic acid (TCA). After 15 minutes on ice the protein was pelleted by centrifugation at 5,000xg for 30 minutes. Residual detergent and TCA were removed by acetone extraction (3x). The pellet was dried in vacuo, and hydrolyzed in 6N HCl containing 0.2% phenol for 16 hours at 115°C. Analysis of the

hydrolysate was performed on a Beckman 121M amino acid analyzer.

Amino acid sequence analysis of intact tissue factor and peptide fragments generated therefrom was performed using an Applied Biosystems (Foster City, CA) gas-phase sequenator. The phenylthiohydantoin (PTH) derivatives liberated at each cycle of sequencing were identified as described by Merrill et al., J. Biol. Chem. 259:10850-10856, 1984. The amino-terminal sequence of intact brain and placental tissue factor was determined using 100-200 μ g of each apoprotein. The protein was precipitated as described above, dissolved in 100% trifluoroacetic acid (TFA) and applied to a GF/C glass filter disc for gas-phase sequencing. Following TCA precipitation and acetone extraction the protein was dissolved in 0.8ml 6M guanidine HCl, 50mM NaHCO₃, pH 8.0. The amino-terminus of the protein was blocked by succinylation with three successive additions of 1mg solid succinic anhydride (Pierce Chemical Co.). After each addition the sample was adjusted to pH 8.0 with 1N NaOH and stirred for 30 minutes. Triton® X-100 was then added to a final concentration of 0.1% and the mixture was dialyzed overnight at 25°C. Following addition of 450 μ l H₂O, the digest was dried (2x) in a vacuum centrifuge. The protein pellet was dissolved in TFA, loaded onto a GF/C glass filter disc and sequenced as described above.

A partial amino acid sequence, spanning residues 211-244, of the carboxy-terminal CNBr peptide (residues 211-263), was obtained by succinylating the intact protein and cleaving at the single methionine residue in the protein, Met²¹⁰, with CNBr, followed by gas-phase sequence analysis. The carboxy-terminal CNBr peptide was prepared from about 60 μ g of the placental apoprotein.

Tryptic peptides of TCA-precipitated placental tissue factor (120 μ g) were also prepared. After

isolation by HPLC, the peptides were sequenced in order to obtain additional sequence information. The TCA-precipitated protein pellet was solubilized in 50 μ l 8M urea and the solution was diluted with 150 μ l 50mM NH₄HCO₃. Trypsin, treated with N-tosyl-L-phenylalanine chloromethyl ketone (TPCK, Cooper Biologicals), was then added at a ratio of 1:25(w/w), trypsin:tissue factor. Digestion was carried out for 24 hours at 37°C and the sample was injected into a Vydac® C-18 HPLC column (0.45x25cm) equilibrated in 0.05% TFA and run at a flow rate of 1ml/min. Peptides were eluted with linear gradients of buffer B (0.05% TFA, 80% acetonitrile) as follows: 0-63 min. (2%-37.5% B), 63-95 min (37.5%-75% B), and 95-105 min (75%-98% B). The elution profile was monitored by absorbance at 210nm and 280nm. The isolated tryptic peptides were sequenced as described above for the intact protein.

Example 2

20 Isolation of Phages λ 10,3 and λ 3,4

Phage λ 10,3 which contains the 2147 bp cDNA insert coding for human tissue factor was isolated by screening a human placental cDNA library cloned into the expression vector phage λ gt11 for human tissue factor coding sequences. The library, which contained about 1.2×10^6 independent placental cDNA-containing recombinants, was purchased from CloneTech (Palo Alto, CA). λ gt11 is a well-known cloning vector (Young and Davis, Science 222:180-182, 1983) which contains the E. coli lacZ gene coding for β -galactosidase. Foreign DNA can be cloned into the EcoRI restriction site of the lacZ gene.

To screen for tissue factor coding sequences in the cloned placental cDNA library, three oligonucleotide hybridization probes were synthesized. These probes which are shown in Fig. 2 hybridized to DNA corresponding to and coding for amino acid

sequences of short peptides from various regions (amino terminal, carboxy terminal and internal) of the tissue factor polypeptide chain which were determined as provided in Example 1. The oligonucleotide probes
5 were synthesized by the phosphoramidite method, using an Applied Biosystems 380A DNA synthesizer. The probes were purified by HPLC on a Nucleogen DEAE 60-7 column (Macherey-Nagel) and radiolabelled at their 5' ends using ^{32}P ATP (Amersham) and T4 polynucleotide
10 kinase (Pharmacia) to a specific activity of approximately 1×10^8 cpm/ μg .

The determination of the partial amino acid sequence of human tissue factor, as provided in Example 1, allowed the construction of the
15 oligonucleotide hybridization probes. With reference to Fig. 2 it can be seen that the probes correspond to DNA coding for three regions of the tissue factor protein. Probe #1 corresponded to the sequence coding for amino acids 24-29 (amino terminal portion of
20 the protein); Probe #3 corresponded to the sequence coding for amino acids 210-215 (carboxy terminal portion of the protein); and probe #2 corresponded to the sequence coding for amino acids 145-149 (internal portion of the polypeptide chain). Probes #1 and 3
25 were both mixtures of 32 oligonucleotides, each 17 bases in length, which were complementary to all the possible coding sequences for each peptide. Probe #2 was a single 45 base deoxyoligonucleotide (45mer) corresponding to an optimal DNA sequence coding for an
30 internal tryptic peptide of human tissue factor. Selection of the optimal coding sequence for the 45mer probe was based on the codon preference in human structural genes described by Lathe, J. Molec. Biol. 183:1-12, 1985.

35 Fig. 2 also depicts that portion of the sequence of the cDNA coding for human tissue factor provided in Formula I in the region of the cDNA fragment which hybridized to Probe #2. The asterisks (*) indicate

base pair mismatches between Probe #2 and the actual tissue factor cDNA coding sequence as determined in Example 3. The overall homology between Probe #2 and the cDNA coding for tissue factor was 75%. Residue
5 158, originally identified as Arg was subsequently identified as Trp.

The placental cDNA library was screened by plating the recombinant phage on a lawn of E. coli K1088 cells grown on agar plates. E. coli K1088, the
10 host for λ gt11, carries the plasmid pMC9 which confers ampicillin resistance and carries the lacI gene (for efficient repression of the lacZ gene). See, Young and Davis, Science 222:778-782, 1983. Screening was performed essentially according to the protocol of
15 Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring, New York, 1982, by transferring recombinant λ gt11 plaques from the agar plates to nitrocellulose filters and hybridizing immobilized phage DNA to the (32 P)-
20 oligonucleotide probes.

Approximately $3-5 \times 10^4$ phage per 85 cm plate which had plated on E. coli K1088 were screened by duplicate filter lifts (Colony/Plaque Screen, New England Nuclear) to the probes. Plaques were first
25 screened by hybridization to Probe #2 at $T_m - 25^\circ\text{C}$ (50°C) in 6X SSC (1X SSC = 0.15 M NaCl, 0.015 M Na_3 citrate, 0.1% sodium dodecyl sulfate (SDS)). The filters were then washed at $T_m - 8^\circ\text{C}$ in 2X SSC and autoradiographed at -70°C with an intensifying screen
30 (DuPont Lightning Plus) for 16 to 40 hours. Phage which gave positive signals with Probe #2 were subsequently plaque-purified and rescreened by hybridization to Probes #1 and #3 as described by Benton and Davis, Science 196:180-182, 1977,
35 to eliminate false positives.

Approximately 2.5×10^6 recombinant λ gt11 phage which contained placental cDNA inserts were screened with Probe #2. Thirty-six potentially positive

plaques were isolated from the library, purified and further screened by hybridization to Probes #1 and #3. In all cases Probe #2 gave significantly stronger hybridization signals than either Probe #1 or #3.

5 Only two of the 36 original recombinant λ phage isolates were reactive with a second probe. The recombinant phage designed λ 10,3 was positive with both Probes #1 and #3, while the recombinant phage designated λ 3,4 only hybridized to Probe #3.

10 The number of recombinant phage clones containing a cDNA coding for human tissue factor was, therefore, estimated to be between 2 and 34 per 2.5×10^6 recombinants. The relative abundance of tissue factor coding sequences in placental cDNA was thus estimated
15 at one human tissue factor cDNA per 7×10^4 to 1×10^6 cDNAs.

The recombinant phage λ 10,3 and λ 3,4 have been successfully propagated in their host organism E. coli K1088. This has allowed the production of large
20 quantity of recombinant phage λ 10,3 and λ 3,4 containing the DNA inserts coding for human tissue factor.

Example 3

25 Sequence and Characterization of the 2147 bp cDNA Coding for Human Tissue Factor

Restriction analysis showed that λ 10,3 contained a 2147 bp DNA insert while λ 3,4 contained a 1616 bp insert. EcoRI, Sau3A, and HindIII restriction fragments of λ 10,3 and λ 3,4 were cloned into the phage
30 cloning vectors M13mp18 and M13mp19 (Messing, Meth. Enzymol. 101:20-78, 1983) to facilitate nucleotide sequence analysis. The sequence of the cDNA inserts in each of the two recombinant phage was determined by the dideoxy chain termination method of Sanger et al.,
35 Proc. Natl. Acad. Sci. 74:5463-5467, 1977, using (35 S)-deoxyadenosine 5'-(α -thio) triphosphate (Amersham; 500 mCi/mmol). Sequencing reactions were analyzed on 6% polyacrylamide-7M urea gels, which were

dried overnight prior to autoradiography. Chain extension reactions were primed using M13 seventeen nucleotide primers which were complementary to positions -20 and -40 relative to the EcoRI site (New England Biolabs; primers #1211 and #1212). Furthermore, eight different 18mer oligonucleotides complementary to human tissue factor cDNA coding sequences were synthesized as described in Example 2 and used to initiate sequencing reactions within large inserts in M13.

Fig. 3 shows a restriction map of the entire cloned 2147 bp cDNA coding for human tissue factor inserted in λ 10,3 and also indicates the corresponding truncated 1616 bp DNA insert in λ 3,4. Both of the cDNAs contained two internal EcoRI sites and the smaller cDNA of λ 3,4 was found to overlap entirely with the larger DNA insert of λ 10,3.

With reference to Fig. 3, the restriction sites used to generate the M13 subclones are indicated at the top of the figure (RI=EcoRI; S=Sau3A; H3=HindIII). The open and solid boxes indicate the coding region for the signal peptide and mature protein, respectively. The second and third lines of Fig. 3 show the relative size and location of the tissue factor cDNA insert in λ 3,4 and λ 10,3, respectively. The wavy lines indicate the length and direction of the DNA sequence determined from the M13 subclones. The open circles indicate where the synthetic primers were used to originate sequencing reactions. These primers correspond to the sequence of the 2147 bp cDNA at nucleotides 283-267, 326-339, 539-527, 821-838, 1075-1039, 1310-1293, 1875-1857 and 1857-1875. The nucleotide sequence was determined for both strands of DNA for 100% of the cDNA coding for the mature tissue factor apoprotein and approximately 82% of the overall cDNA sequences.

The nucleotide sequence of the entire 2147 bp cDNA from λ 10,3 coding for human tissue factor is pro-

vided in Formula I. With reference to Formula I, the 2147 bp cDNA contains a single long open reading frame of about 885 bp which extends from an ATG initiation codon at nucleotides 112-114 to a TAA termination
5 codon at nucleotides 997-999. The open reading frame codes for a preprotein of 295 amino acids which is the precursor for the 263 amino acid mature human tissue factor apoprotein. The entire preprotein sequence which was deduced from the DNA sequence is also
10 provided in Formula I. Based on a comparison to the amino terminal sequence of the mature human tissue factor (Ser-Gly-Thr-Thr-Asn) as determined in Example 1 it was seen that the preprotein contains a 32 amino acid leader sequence or signal peptide.

15 As shown in Formula I, the 5' untranslated region of the mRNA corresponding to the 2147 bp cDNA is very GC rich (72% G + C) as are a number of other eukaryotic signal peptide regions (see, e.g., Ohno et al., Nature 325:161-166, 1987). The 3'
20 untranslated sequence of 1147 nucleotides following the coding portion was slightly AT rich (63% A+T). The sequence AATAAA at nucleotides 2121-2126 apparently is the mRNA polyadenylation sequence since it is followed by a sequence of six A residues at
25 nucleotides 2142-2147.

The accuracy of the DNA sequence coding for tissue factor provided in Formula I was evaluated by comparing the amino acid sequence of the protein predicted from the nucleotide sequence of the open
30 reading frame of the cDNA coding for tissue factor to the amino acid sequence of intact tissue factor, and tryptic digests thereof, purified from human brain and placenta and sequenced by protein sequencing techniques as provided in Example 1.

35 The portions of the amino acid sequence of tissue factor apoprotein predicted from the sequence of the open reading frame of the cDNA coding for tissue which were confirmed by amino acid sequence analysis as

described above are underlined in Formula I. A total of 71.5% of the coding region for the mature protein was confirmed by amino acid sequence analysis and all peptide sequences were matched to predicted peptides. With the exception of residue 208 which was predicted to be glutamic acid from the DNA sequence of the cDNA inserts of both λ 10,3 and λ 3,4 and glycine based on gas phase sequencing of the peptide encompassing residues 202-215, all of the remaining 262 amino acid residues determined by protein or peptide sequencing agreed with the protein sequence provided in Formula I which was predicted from the sequence of the cDNA coding for tissue factor. The complete amino acid sequence of human tissue factor was not known previously. Thus, the 2147 bp cDNA fragment coding for human tissue factor provides for the synthesis of substantially pure human tissue factor having an amino acid sequence as provided in Formula I.

Example 4

20 Further Characterization the of Human Tissue Factor Apoprotein

The amino-terminal sequence of human tissue factor was determined as described in Example 1 for the apoprotein purified from both brain and placenta. The two preparations gave identical sequences to each other and to the amino acid sequence predicted from the cDNA sequence of the 2147 bp cDNA fragment. Amino acid residues 1-22 of brain tissue factor and residues 1-38 of the placental protein were identical to the predicted sequence of tissue factor provided in Formula I. Each cycle of amino acid sequencing of the intact proteins yielded two PTH amino acids giving overlapping sequences that were out of phase by two residues. This interpretation is consistent with the primary structure as derived from the cDNA sequence (Formula I). Thus, approximately half of the tissue factor apoproteins isolated from brain and placental tissue lacked the first two amino acids. No PTH amino

acid was found at position 11, which is believed to be due to glycosylation of the Asn residue at this consensus N-linked glycosylation site (Asn-X-Ser/Thr). (See e.g., Marshall, Biochem. Soc. Symp. 40:17-26, 1974).

The entire amino acid sequence for mature tissue factor shown in Formula I provides for a molecular weight, M_r , of the substantially pure tissue factor apoprotein of about 29,600, which is smaller than the M_r of 44-46,000 previously estimated for human tissue factor by SDS-PAGE by Broze et al., J. Biol. Chem. 260:10917-10920, 1985; Guha et al., Proc. Natl. Acad. Sci. 83:299-302, 1986. To examine this discrepancy, tissue factor purified from brain and/or placenta was enzymatically and chemically deglycosylated and the M_r of the resulting apoprotein was determined.

Placental tissue factor (5 μ g) was chemically deglycosylated by the method of Edge et al., Anal. Biochem. 118:131-137, 1981, using trifluoromethanesulfonic acid (TFMS). The protein was precipitated with 5 volumes of acetone and pelleted at 5000xg for 30 minutes, after which it was dried in vacuo; 20 μ l of anisole and 40 μ l TFMS were then added. The tube was flushed with N_2 for 30 seconds, sealed and incubated for 3 hours on ice. The sample was then extracted twice with 3ml ether:hexane (9:1), with 5 μ l pyridine added to facilitate protein precipitation. Finally, the resulting pellet was extracted once with acetone.

Asparagine-linked carbohydrate was enzymatically cleaved from tissue factor by digestion with endoglycosidase F as described by Steub et al., Biochemistry 24:3587-3592, 1985. Human placental tissue factor (5 μ g) was precipitated with acetone as described above. The protein pellet was then dissolved in 40 μ l of a buffer containing 0.5% 8-octyl-D-glucopyranoside, 20mM EDTA, and 50mM sodium acetate at pH 6.1, and endoglycosidase F (0.1 unit, Boehringer Mannheim, grade II) was added. After

incubation for 16 hours at 37°C, the reaction was terminated by acetone precipitation. The products of chemical and enzymatic deglycosylation were then analyzed by PAGE on the SDS-urea gel system of Swank and Munkres, Anal. Biochem. 39:462-477, 1971.

Untreated tissue factor migrated with an apparent M_r of 42,000 in the SDS/urea PAGE system of Swank and Munkres. This value differed significantly from the apparent M_r of 46,000 observed in the Laemmli SDS-PAGE system and suggested anomalous detergent binding. The TFMS deglycosylated protein gave an apparent M_r of 34,500 in the Swank and Munkres system while the value of the enzymatically deglycosylated material was 33,500. Thus, carbohydrate contributes about 7,500-8,500 daltons to the apparent M_r of tissue factor. It is believed that all the carbohydrate is N-linked (to Asn) since chemical and enzymatic digestions gave essentially the same result.

The complete sequence of human tissue factor provided in Formula I has also been examined for homology with 4668 sequences in the protein sequence database of the National Biomedical Research Foundation, Washington, D.C. The homology search, which was performed using the FASTP program of Lipman et al., Science 227:1435-1441, 1985, revealed no significant amino acid sequence homology between human tissue factor and any protein in the database. In particular, no similarities in primary amino acid sequence were observed between the sequence of tissue factor provided in Formula I and the known amino acid sequences of other clotting proteins, such as thrombomodulin, factor VII, and factor V.

Example 5 (Fig. 4)

Construction of Plasmid pKS-2B

Phage λ 10,3 DNA was digested with the restriction enzymes KpnI and SstI which yielded a DNA fragment comprising the entire 2147 bp cDNA insert coding for

tissue factor plus approximately 1000 bp of λ phage DNA from each side of the insert. The approximately 4.15 kb KpnI/SstI fragment so obtained was then cloned into the lacZ gene of plasmid pUC19 (Norrand et al., 5 Gene 26:101, 1983; Yanisch-Perron et al., Gene 33:103, 1985) which had been digested with KpnI and SstI. The resulting recombinant plasmid pKS-2B containing the tissue factor gene was then used to transform E. coli 71-18, a host for pUC19. E. coli 71-18/pKS-2B 10 transformants have provided an excellent source of the tissue factor gene for further study and for subsequent construction of expression vectors for production of mature human tissue factor apoprotein (Example 7) and soluble human tissue factor (Example 15 8) and for localization and characterization of the genomic human tissue factor gene (Example 6).

Example 6

Localization of Human Tissue Factor Coding Sequences in Genomic DNA

20 Tissue factor coding sequences were localized to an approximately 9.5 kb fragment of genomic DNA by Southern blotting hybridization techniques. This procedure which involves transfer of DNA restriction fragments from an agarose gel after electrophoresis to 25 an appropriate membrane, followed by hybridization with a specific probe, has been used in numerous studies to analyze genome organization and function.

A 1.25 kb Sau3A/HindIII DNA fragment comprising nucleotides 90 to 1340 of the 2147 bp tissue factor 30 cDNA was obtained from plasmid pKS-2B. This 1.25 kb fragment which contained the entire human tissue factor open reading frame (ORF) plus about 110 bp of 5'-untranslated and 343 bp of 3'- untranslated sequences was used to probe for tissue factor coding 35 sequences in total human genomic DNA.

Total human placental genomic DNA was digested with a panel of restriction enzymes. Three (3) μ g of each digest were electrophoresed on a 10% agarose gel

and then treated with 0.25 M HCl for 15 min. This brief acid treatment following electrophoresis partially depurinated the DNA, breaking it down into smaller pieces that were more readily transferable to the membrane. The acid treatment step was especially important for quantitative transfer of DNA fragments which were more than 5kb in length.

Following acid treatment, the gel was briefly rinsed in distilled water and overlaid with a nylon transfer membrane (Zeta Probe[®], Bio Rad). Transfer of the restriction fragments to the membrane was performed by incubation overnight in the presence of a solution of 0.4N NaOH. The transferred DNA became covalently bound to the membrane.

The 1.25 kb Sau3A/HindIII fragment from pKS-2B was used to probe the digested genomic DNA for tissue factor coding sequences. Prior to hybridization the fragment was radiolabelled to a very high specific activity of about 5×10^8 cpm/ μ g using the random priming methods described by Feinberg and Vogelstein, Anal. Biochem. 132:6, 1983; Anal. Biochem. 137:266, 1984.

Southern blotting (hybridization) of the labelled 1.25 kb fragment to the genomic restriction fragments bound to the membrane was carried out according to the technique described in Bio Rad Bulletin 1234. Prior to hybridization the membrane was treated at 47°C with 50% formamide; 4xSSPE (20xSSPE=3.6M NaCl, 0.2M sodium phosphate, pH 7.0, 0.2M EDTA); 1% SDS; 0.5% Blotto; and 0.5mg/ml carrier DNA (salmon sperm). 10% Blotto was prepared by suspending 10g of nonfat powdered milk (Diploma) in 100 ml sterile deionized water and adding sodium azide to a final concentration of 0.2%. The carrier DNA in the prehybridization step was used to minimize non-specific hybridization.

Hybridization was carried out using a hybridization cocktail containing the radiolabelled 1.25 kb tissue factor DNA probe in 47% formamide; 10%

dextran sulfate; 3xSSPE; 1% SDS and 0.5% Blotto.

Immediately prior to hybridization, the probe was fragmented and denatured by dissolving the radio-labelled 1.25 kb fragment in 0.2M NaOH. Excess

5 carrier DNA was added and the mixture was agitated and centrifuged briefly. The mixture was heated for about 5 minutes at 100°C prior to addition to the hybridization cocktail.

The membrane containing the digested human
10 genomic DNA was incubated with the probe/hybridization cocktail overnight at 47°C ($T_m - 11^\circ\text{C}$). The blot was then washed at room temperature for 15 min. with 2xSSC/0.1% SDS followed by washing for 15 min. with 0.5xSSC/0.1% SDS. The blot was further washed at 50°C
15 ($T_m - 5^\circ\text{C}$) for 15 min. with 0.1 SSC/0.1% SDS. Following washing, the blot was exposed overnight at -70°C to an X-ray film (XAR X-Omat, Kodak) with one intensifying screen.

Fig. 5 is a Southern blot which shows the results
20 of hybridization of the 1.25 kb DNA fragment containing the tissue factor coding sequences to various restriction digests of the placental genomic DNA. Lanes 1 and 8 are control digests: phage λ DNA digested with HaeIII or HindIII. Lane 2 is Ball
25 digested human DNA; Lane 3 is an EcoRI digest, lane 4 is a HindIII digest; Lane 5 is a PstI digest; Lane 6 is a Sau 3A digest; and Lane 7 is a SspI digest. The right side of Fig. 4 shows the electrophoretic mobility of DNA of defined lengths (kb).

30 With reference to Fig. 5 it can be seen that the 1.25 kb tissue factor probe hybridized to two HindIII fragments of 7.0 and 2.546 kb, respectively; to four PstI fragments of 4.2, 3.0, 1.5 and 0.7 kb, respectively; and to four SspI fragments of 4.5, 3.8,
35 0.98 and 0.35, respectively. From this information the size of the chromosomal gene encompassing the DNA coding for tissue factor was determined to be about

9.5 kb. It is also believed that the gene contains at least 3 introns.

Example 7

Expression of Human Tissue Factor

5 Several host systems have been selected for expression of the structural gene for human tissue factor, e.g. E. coli, CHO cells and insect cells. Expression in these host systems can be accomplished using known techniques to construct expression vectors
10 for expressing tissue factor in the selected host, e.g. M13/pUC vectors for E. coli, mammalian shuttle vectors for CHO cells and baculovirus for insect cells. Expression of tissue factor can also be accomplished in yeast cells using yeast expression vectors,
15 e.g. CYC1, YCpCYC1, YRpCYC1 and dYeCEN3.

(a) Expression of Biologically Active Human Tissue Factor in E. coli

(1) Construction of plasmid pTL8FQ

Plasmid pTL8FQ, which in a suitable host, e.g.
20 E. coli 71-18, expresses the human tissue factor apoprotein, was constructed as follows:

With reference to Fig. 4, the 1.25 kb SauIIIA/HindIII fragment from pKS-2B, obtained as in Example 6, was ligated to BamHI/HindIII digested DNA of the
25 cloning vector M13 mp19 to produce the vector M13/LB2TF.

Using the TFAD oligonucleotide sequence shown in Fig. 6C, six bases were then inserted between nucleotides 201 and 202 of the tissue factor coding
30 region in single-stranded DNA from M13/LB2TF by site specific mutagenesis to create a PstI cleavage site in the gene. This latter construct, designated M13/LB3TF (Fig. 6A), was digested with PstI and HindIII and ligated to PstI/HindIII digested pUC19 DNA to produce
35 plasmid pLB4TF, as shown in Fig. 6A.

Next, using the oligonucleotide sequence designated G8PST (Fig. 6C), a PstI site was inserted

by site specific mutagenesis into single stranded DNA from bacteriophage tgl31 (a derivative of phage M13 which contains multiple cloning sites) just downstream from the M13 gene VIII leader sequence to form
5 M13/TL131P (Fig. 6B). A 100 bp SnaBI/PstI fragment from M13/TL131P was then inserted into the PstI/SmaI site of plasmid pLB4TF to produce plasmid pTL8TF, which contained the DNA coding for the M13 gene VIII leader sequence together with the structural gene for
10 human tissue factor (Fig. 7). Plasmid pTL8TF was then digested with SstI and HindIII to produce a 1252 bp restriction fragment containing the gene VIII leader and tissue factor structural DNA sequences. This fragment was then isolated by agarose gel
15 electrophoresis and cloned into plasmid pTL131Q which had been linearized with SstI and HindIII (Fig. 7).

Plasmid pTL131Q had been constructed by inserting tgl31 DNA into the unique PvuII site of plasmid ptac-12, which had been obtained from Dr. John Brosius.
20 Plasmid ptac-12 is a pBR322 derivative and contains the hybrid "tac" (trp/lac) promoter, as disclosed in Amann et al., Gene 25:167-178, 1983. The DNA of phage tgl31 was cleaved with BglII and EcoRI converted to blunt-ends using the Klenow fragment of DNA polymerase
25 I from E. coli. Insertion of this DNA into the PvuII site of ptac-12 destroyed the BglII site but restored the EcoRI site in the recombinant pTL131Q. Plasmid pTL131Q now contained the tac promoter, which is inducible with isopropylthiogalactoside (IPTG).

30 Insertion of the 1252 bp fragment from pTL8TF into pTL131Q resulted in the recombinant expression vector pTL8FQ (Fig. 7), which in a suitable host, e.g. E. coli 71-18, expresses the human tissue factor apoprotein.

35 (2) Expression of Human Tissue Factor in E. coli/pTL8FQ Transformants

Plasmid pTL8FQ was then used to transform E. coli 71-18. Because the human tissue factor gene is under

the control of the tac promoter in the plasmid, IPTG was used to induce maximum expression of human tissue factor in E. coli 71-18/pTL8FQ transformants. After induction, the cells were sonicated and assayed for tissue factor activity using the two stage clotting assay as in Example 1. Transformant extracts demonstrated a specific activity of tissue factor of about 6×10^4 units/mg protein which corresponds to about 1ng tissue factor per ml culture. Purified human tissue factor protein as prepared in Example 1 was used as a standard for comparison.

As shown in Fig. 8, Western blotting analysis with the anti-human tissue factor monoclonal antibody demonstrated the presence of proteins which specifically reacted with the monoclonal anti-tissue factor antibody. Molecular weight standards were run in Lane 1; Lane 2 - total E. coli 71-18/pTL8FQ transformant cell extracts; Lane 3 - Supernatant obtained following centrifugation of sonicated E. coli 71-18/pTL8FQ transformant cells; Lane 4 - Pellet obtained following centrifugation of E. coli 71-18/pTL8FQ transformant cells. The approximate molecular weights of the two bands were 35,000 and 33,000, respectively. The ratio of the 35K to 33K bands was about 5:1. The larger protein is believed to be a protein which contains the M13 gene VIII leader sequence.

(b) Construction of a Human Tissue Factor
Expression Vector For Use in Mammalian Cells

A shuttle vector, pMAM (obtained from CloneTech, cat. #6100-1), capable of transforming E. coli and mammalian cells, such as CHO cells, was selected for construction of a mammalian vector which expresses human tissue factor in a suitable mammalian host cell, e.g. CHO cells. The pMAM vector contains a number of important features which make it suitable for such purposes including the mouse mammary tumor virus (MMTV) promoter, which allows for dexamethasone

regulation of transcription and multiple cloning sites adjacent the MMTV promoter, as well as genes for E. coli guanine phosphoribosyl transferase, which codes for a selectable marker to indicate transformation of mammalian cells, and ampicillin-resistance, a selectable marker to detect the plasmid in E. coli transformants. Finally, pMAM also contains a SV₄₀ polyadenylation site which allows for synthesis of proteins in mammalian cells.

Fig. 9 depicts the pMAM/TF vector which is usable to express human tissue factor in mammalian cells. The vector was constructed by cloning the pKS-2B Sau3A/HindIII fragment described in Example 6 into pMAM as follows: pKS-2B DNA was first digested with HindIII, followed by the addition of XhoI linkers to the HindIII cleavage site. Next, the DNA was digested with SstI, followed by addition of XbaI linkers to the SstI cleavage site. The resulting fragment was cloned into pMAM DNA which had been linearized by cleavage with NheI and XhoI to produce pMAM/TF (Fig. 9).

E. coli XL-1 blue cells (obtained from Stratagene Corp.) are transformed with pMAM/TF. Ampicillin-resistant XL-1/pMAM/TF transformants are screened for the presence of the human tissue factor gene. XL-1 blue transformants which contain the tissue factor coding sequences are selected, the plasmid amplified and obtained for transforming suitable CHO host cells (which lack the gene coding for guanine phosphoribosyl transferase). CHO/pMAM/TF transformants are selected in a medium in which only the cells which have acquired the functional guanine phosphoribosyl transferase can grow, e.g. HAT medium. Transformants growing in the selection medium are then tested for the presence of the human tissue factor DNA sequences and for production of human tissue factor protein.

Example 8Expression Of Soluble
Human Tissue Factor Protein

With reference to Fig. 10, the Sau3a/HindIII
5 1.25 kb DNA fragment containing the entire tissue
factor open reading frame (ORF) described in Example 6
was ligated to BamHI/HindIII digested pUC19. The
resulting plasmid was then linearized by digestion
first with HindIII, followed by a partial digestion
10 with SspI, which produced a cut within the tissue
factor ORF at a site approximately corresponding to
amino acids 219-220 of the apoprotein sequence
(reading from the N-terminal). Following
electrophoresis of the digested DNA on an agarose gel,
15 a 3,447 bp fragment was obtained which contains a DNA
sequence coding for the extracellular domain
(approximately amino acids 1-219/220) of the mature
apoprotein. Recessed ends in the fragment were filled
in using the Klenow fragment of E. coli DNA polymerase
20 I. XbaI linkers (obtained from New England Biolabs,
Cat. #1062), which contained nonsense codons in three
reading frames, were ligated to the fragment. The
fragment was then digested with XbaI and religated to
produce plasmid pLB5TF (Fig. 10).

25 The XbaI linker adjacent to the 3' side of the
truncated tissue factor ORF introduces a stop codon in
the plasmid just downstream from the DNA coding for
the C-terminal end of the tissue factor extracellular
domain. Plasmid pLB5TF thus codes for a truncated
30 soluble human tissue factor which comprises the
extracellular domain. In order to amplify the
truncated tissue factor coding sequences, plasmid
pLB5TF was inserted into and replicated by E. coli
71-18 cells. Plasmid pLB5TF, however, is not an
35 expression vector and E. coli 71-18/pLB5TF
transformants do not produce soluble tissue factor.

An expression vector pLB6TF which in a trans-
formant host expressed soluble human tissue factor

comprising the extracellular domain was produced as follows (see Fig. 11):

Plasmid pTL8FQ described in Example 7 was digested with Eco0109 and AccI and electrophoresed. A 500 bp fragment which comprises the DNA sequence coding for the leader sequence of the bacteriophage M13 gene VIII product, the N-terminal amino acids 1-34 of the mature human tissue factor apoprotein (plus an additional ala-asp at the N-terminus) the tac promoter region and a portion of vector DNA was isolated from the agarose gel following electrophoresis and ligated to a DNA fragment from pLB5TF which had been digested with Eco0109 and AccI. The resulting plasmid pLB6TF contained the tac promoter and a DNA sequence coding for the gene VIII leader attached to the DNA sequences coding for the extracellular domain of mature human tissue factor. pLB6TF is an expression vector which, in a suitable host, such as E. coli 71-18, produced soluble active human tissue factor comprising the extracellular domain of the mature human tissue factor apoprotein.

Since the truncated soluble tissue factor expression is under the control of the tac promoter, E. coli 71-18/pLB6TF transformants were induced with IPTG in order to maximize expression of the soluble human tissue factor. The transformant cells were harvested in late log phase and converted to spheroplasts and a supernatant fraction containing periplasmic proteins using standard techniques. Tissue factor activity in the spheroplasts and in the supernatant fraction containing periplasmic proteins (periplasmic fraction) from E. coli 71-18/pLB6TF transformants was assayed using the two stage clotting assay described by Bach et al., J. Biol. Chem. 256:8324-8331, 1981, as discussed in Example 1.

A low amount of tissue factor procoagulant activity was detectable in both the spheroplast and periplasmic fractions. The activity in spheroplasts

was about 100 times higher than the activity in the periplasmic fraction. To increase the activity of the soluble tissue factor in the periplasmic fraction, 100 ml of mixed brain lipid (10 mg/ml) or 100 ml of 0.25% deoxycholate was added to 900 ml of the periplasmic fraction and the mixture was dialyzed overnight against 250 ml of 50 mM Tris-HCL pH 7.5, 100mM NaCl. Aliquots (20 ml) were analyzed for tissue factor activity by the two stage clotting assay.

Relipidation of the periplasmic fraction resulted in an increase in tissue factor activity to approximately the level found in the spheroplast fraction.

The soluble tissue factor could be purified from the periplasmic fraction by immunoaffinity chromatography on an immunoabsorbent column prepared with the monoclonal anti-human tissue factor antibody as described in Example 1. Soluble tissue factor was eluted from the column with 0.1M glycine-HCl, pH2.1.

The soluble human tissue factor present in the spheroplasts and in the periplasmic fraction, as well as antibody-purified soluble tissue factor, was also analyzed by Western blot analysis using the monoclonal antibody to human tissue factor. As shown in Fig. 12A, the periplasmic fraction contained a single protein with a molecular weight of about 30,000 which bound the antibody, while two proteins of molecular weights of about 32,500 and 30,000 daltons, respectively, were found in the spheroplasts. The samples were applied to the gel as follows: Lane 1, MW standards; Lane 2, spheroplast fraction; Lane 3, periplasmic fraction; Lanes 4-6, breakthrough fraction from immunoabsorbent column; Lane 8, soluble human tissue factor eluted from immunoabsorbent column with glycine-HCl. The larger protein is believed to be a preprotein containing the M13 gene VIII leader sequence which is subsequently removed from the soluble human tissue factor upon export of the protein into the periplasmic space. The antigenic material

appeared to be equally distributed between the spheroplasts and periplasmic fraction. Furthermore, the soluble tissue factor protein is readily isolatable on the immunoabsorbent column.

- 5 Fig. 12B is a second Western blot of the purified product from E. coli 71-18/pLB6TF transformants. Lane 1 contained molecular weight standards; Lanes 2 and 3, purified soluble tissue factor. The procoagulant activity of the product was determined to be about
- 10 1.9×10^4 units/mg protein. The soluble tissue factor is thus a much less active procoagulant than the intact apoprotein. The soluble protein, however, does bind to Factor VII and can inhibit the binding of the intact apoprotein to Factor VII.

Claims

1. Recombinant cloning vector replicable in a host containing within its genome a DNA insert the sequence of which codes for human tissue factor or a portion thereof.
5
2. A host organism containing a replicable recombinant cloning vector therein, said vector containing a DNA insert therein the sequence of which codes for human tissue factor or a portion thereof.
10
3. A recombinant cloning vector according to claim 1 in which the vector is selected from the group consisting of plasmids, cosmids, bacteriophage, other vectors in replicable in bacteria and vectors replicable in eukaryotes.
15
4. A recombinant cloning vector according to claim 3 in which the vectors replicable in eukaryotic hosts are selected from the group consisting of vaccinia virus, bovine papilloma virus, simian virus₄₀, yeast vectors and baculovirus.
20
5. A host organism according to claim 2 in which the host organism is selected from the group consisting of bacteria, yeast, insect cells, animal cells and human cells.
- 25 6. Recombinant cloning vector according to claim 1 in which the recombinant cloning vector is selected from the group consisting of phage λ 10,3, phage λ 3,4 and plasmid pKS-2B.
- 30 7. Recombinant cloning vector according to claim 1 in which the recombinant cloning vector is phage λ 10,3.

8. Recombinant cloning vector according to claim 1 in which the recombinant cloning vector is plasmid pKS-2B.
9. A host organism according to claim 2 in which the organism is E. coli strain K1088.
10. A host organism according to claim 2 in which the organism is E. coli strain 71-18.
11. A host organism according to claim 9 in which the recombinant cloning vector is phage λ 10,3.
12. A host organism according to claim 9 in which the recombinant cloning vector is phage λ 3,4.
13. A host organism according to claim 10 in which the recombinant cloning vector is plasmid pKS-2B.
14. A 2147 base pair cDNA fragment coding for human tissue factor, which comprises a nucleotide sequence as provided in Formula I, having an open reading frame extending from an ATG initiation codon at nucleotides 112-114 to a TAA termination codon at nucleotides 997-999, the open reading frame coding for a preprotein of human tissue factor being a single polypeptide chain and having a sequence of 295 amino acids, said preprotein being postrationally cleaved to substantially pure mature tissue factor which is a single polypeptide chain having an amino acid sequence of 263 amino as provided in Formula I.
15. Substantially pure human tissue factor apoprotein or a functional portion thereof having an amino acid sequence as provided in Formula I.

16. An oligonucleotide probe for identification of DNA coding for human tissue factor, the nucleotide sequence of which corresponds to a sequence of DNA coding for amino acids 24 through 29 of substantially pure human tissue factor having an amino acid sequence as provided in Formula I.
17. An oligonucleotide probe for identification of DNA coding for human tissue factor, the nucleotide sequence of which corresponds to a sequence of DNA coding for amino acids 145 through 159 of substantially pure human tissue factor having an amino acid sequence as provided in Formula I.
18. An oligonucleotide probe for identification of DNA coding for human tissue factor, the nucleotide sequence of which corresponds to a sequence of DNA coding for amino acids 210 through 215 of substantially pure human tissue factor having an amino acid sequence as provided in Formula I.
19. Recombinant cloning vector which in a suitable host will express a gene coding for human tissue factor apoprotein or a functional portion thereof.
20. A host organism which produces human tissue factor apoprotein or a functional portion thereof, the host being transformed by a recombinant cloning vector containing a DNA insert which codes for human tissue factor or a functional portion thereof.
21. Recombinant vector according to claim 19 in which the vector is selected from the group consisting

of plasmids, cosmids, bacteriophage, other vectors in replicable in bacteria and vectors replicable in eukaryotes.

22. Recombinant vector according to claim 22 in which
5 the vectors replicable in eukaryotic hosts are selected from the group consisting of vaccinia virus, bovine papilloma virus, simian virus₄₀, yeast vectors and baculovirus.
23. A host organism according to claim 20 in which
10 the host organism is selected from the group consisting of bacteria, yeast, insect cells, animal cells and human cells.
24. Recombinant vector according to claim 19 in which the vector is pTL8FQ.
- 15 25. Recombinant vector according to claim 19 in which the vector is pMAM/TF.
26. A host organism according to claim 20 in which the host is E. coli strain 71-18.
27. A host organism according to claim 20 in which
20 the host is E. coli XL-1 blue.
28. A host organism according to claim 20 in which the host is CHO cells.
29. Recombinant cloning vector according to claim 19 for expression of soluble human tissue factor or
25 a functional portion thereof.
30. Recombinant cloning vector according to claim 29 in which the soluble human tissue factor comprises the amino terminal extracellular domain of mature human tissue factor apoprotein.

- 5 31. Recombinant cloning vector according to claim 29 in which the soluble human tissue factor comprises the N-terminal amino acids 1-219/220 of mature human tissue factor, the sequence of which is provided in Formula I.
32. Recombinant cloning vector according to claim 31, in which the recombinant vector is pLB6TF.
33. A host organism according to claim 20 which produces soluble human tissue factor.
- 10 34. A host organism according to claim 33 in which the soluble tissue factor comprises the extracellular domain of mature human tissue factor apoprotein or a functional portion thereof.
- 15 35. A host organism according to claim 33 in which the soluble tissue factor comprises the N-terminal amino acids 1-219/220 of mature human tissue factor as provided in Formula I.
- 20 36. A host organism according to claim 35 in which the host is E. coli strain 71-18.
- 25 37. Substantially pure soluble human tissue factor, wherein the soluble tissue factor comprises the extracellular domain of mature human tissue factor or a functional portion thereof, the sequence of which is provided in formula I.
- 30 38. Soluble human tissue factor according to claim 37 in which the soluble tissue factor comprises the N-terminal amino acids 1-219/220 of mature human tissue factor, the sequence of which is provided in Formula I.

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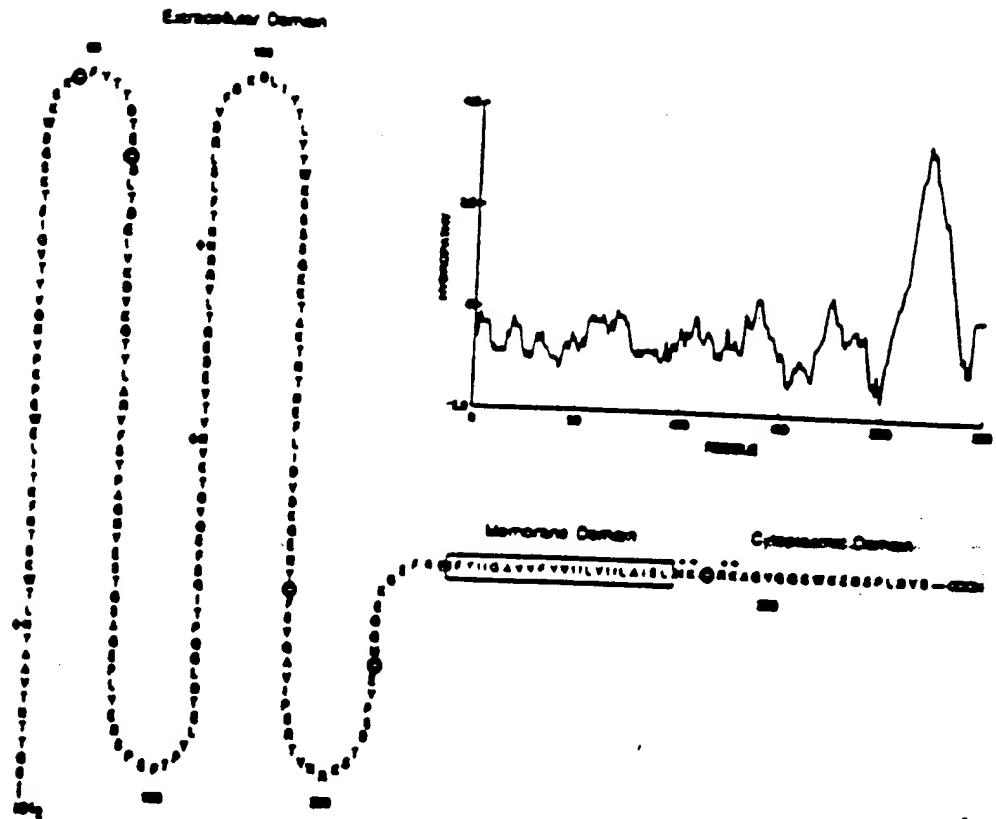


Figure 1

mixture of 32

5'-GAT-GTT-TTT-GCC-AAG-GAC-TTA-ATT-TAT-ACA-CTT-TAT-TAT-TGG-AAA-3'

3'-TAC-CCX-GT_C⁶-CT_C⁶-TT_C⁶-CC-5' mixture of 32

Figure 2

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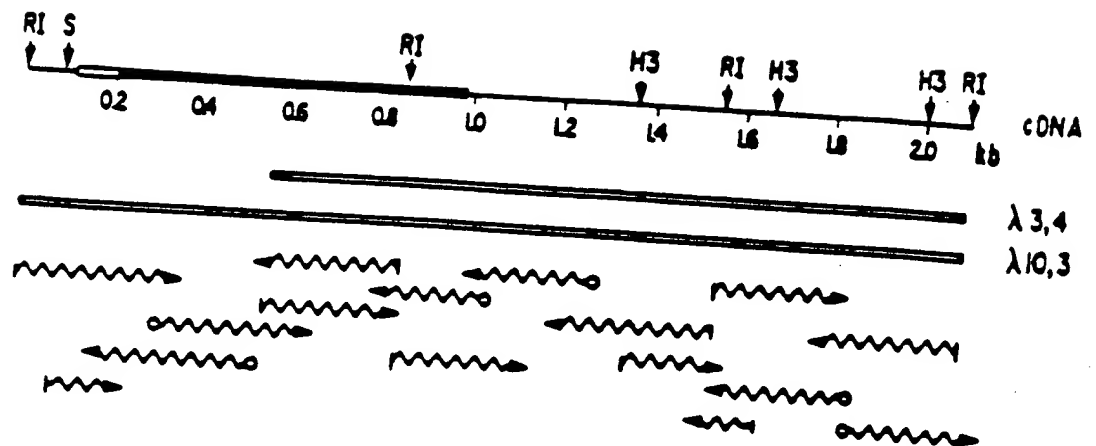


Figure 3

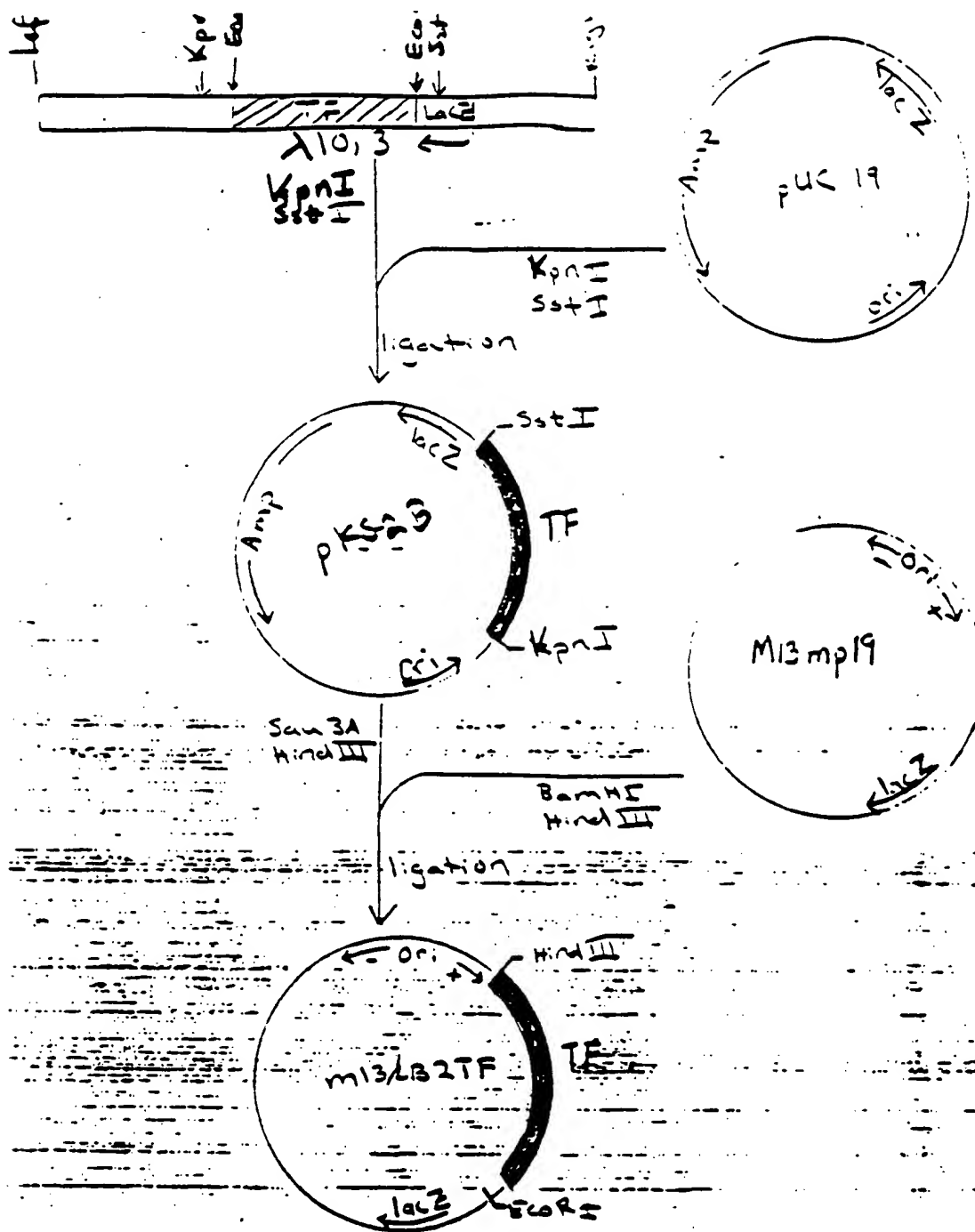


Fig 4

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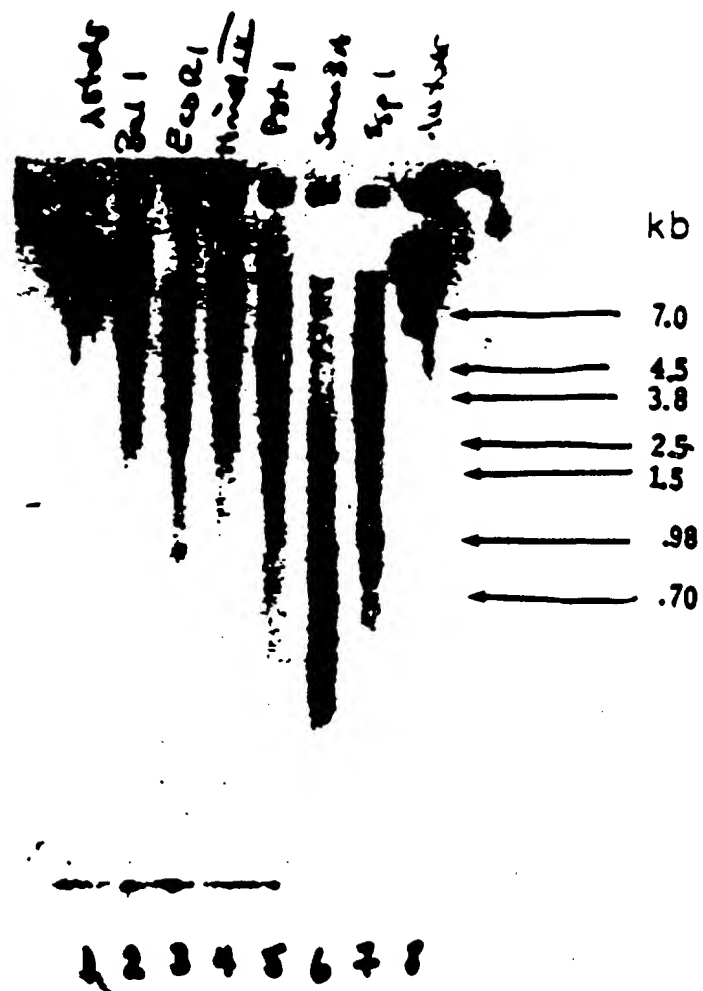


Fig. 5

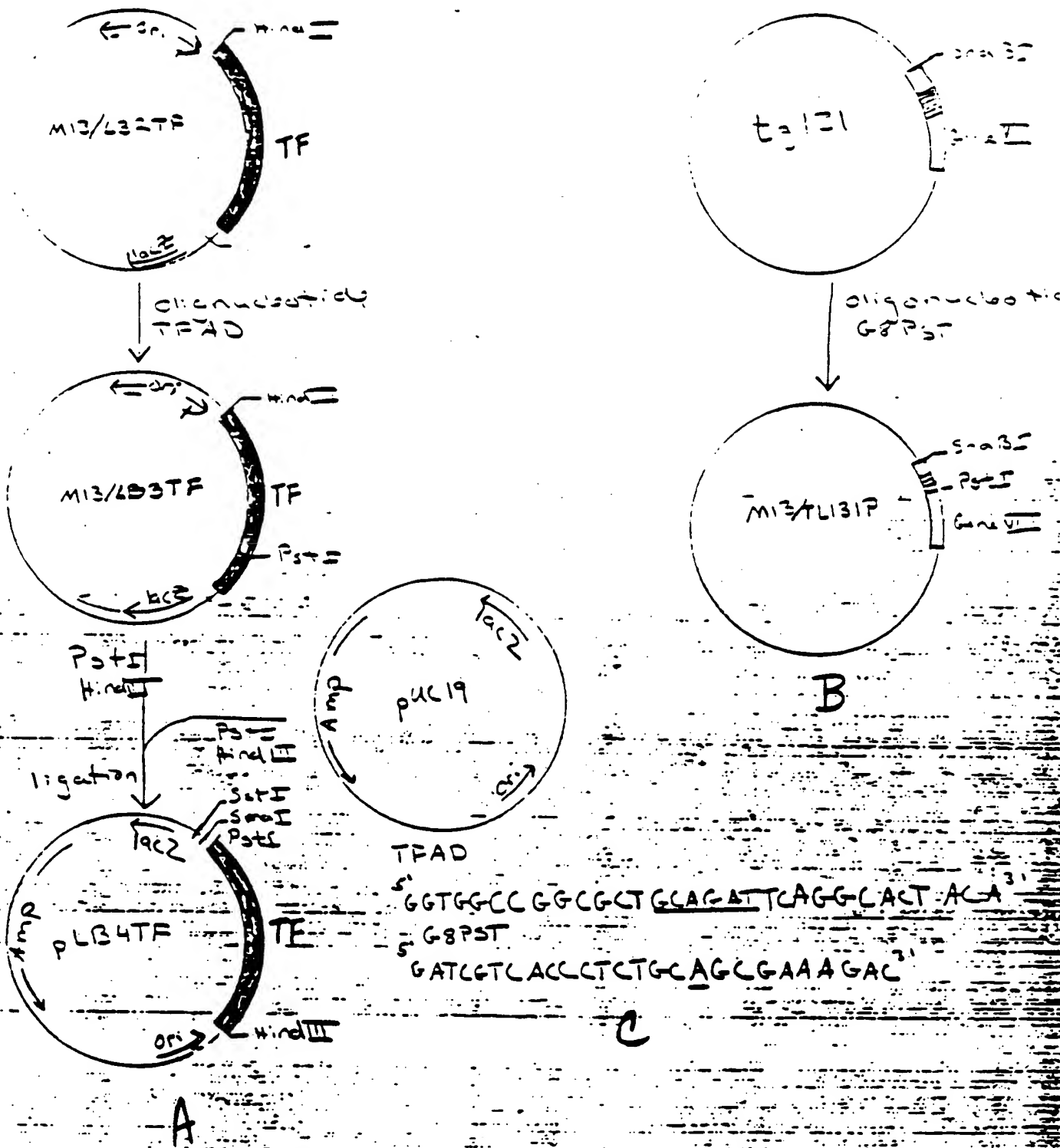


Fig. 6

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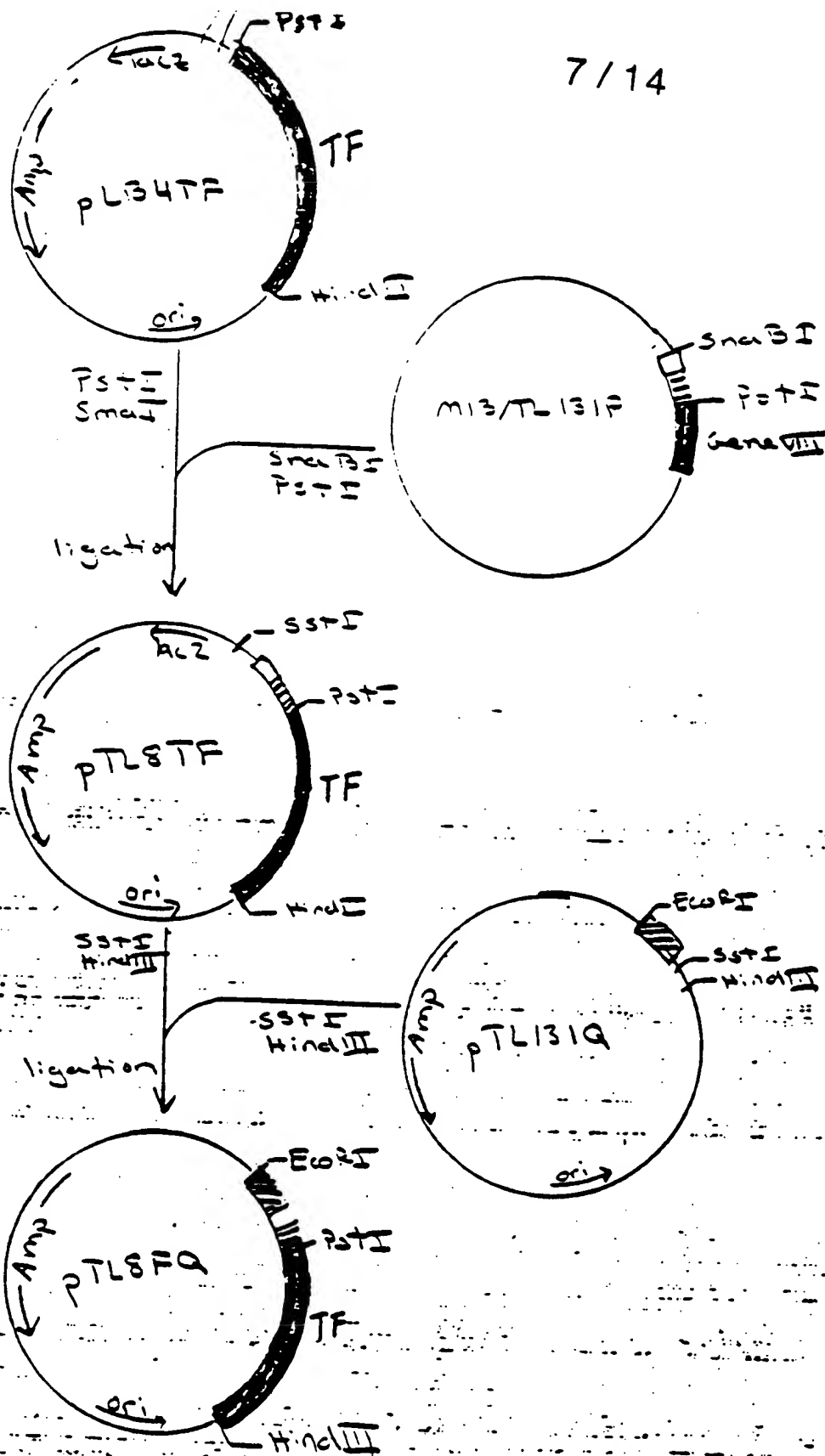


Fig. 7

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1 2 3 4

Fig. 8

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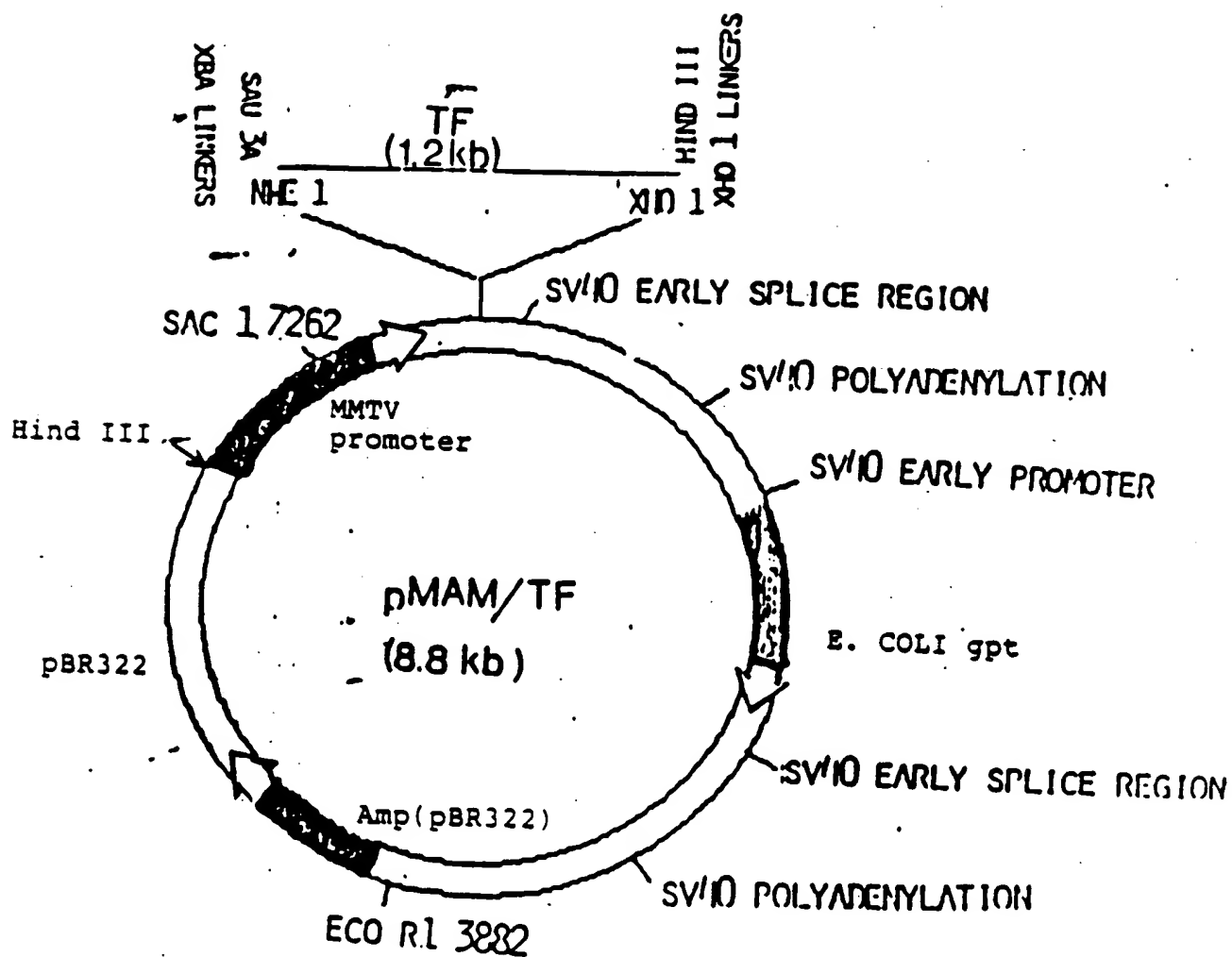


Fig. 9

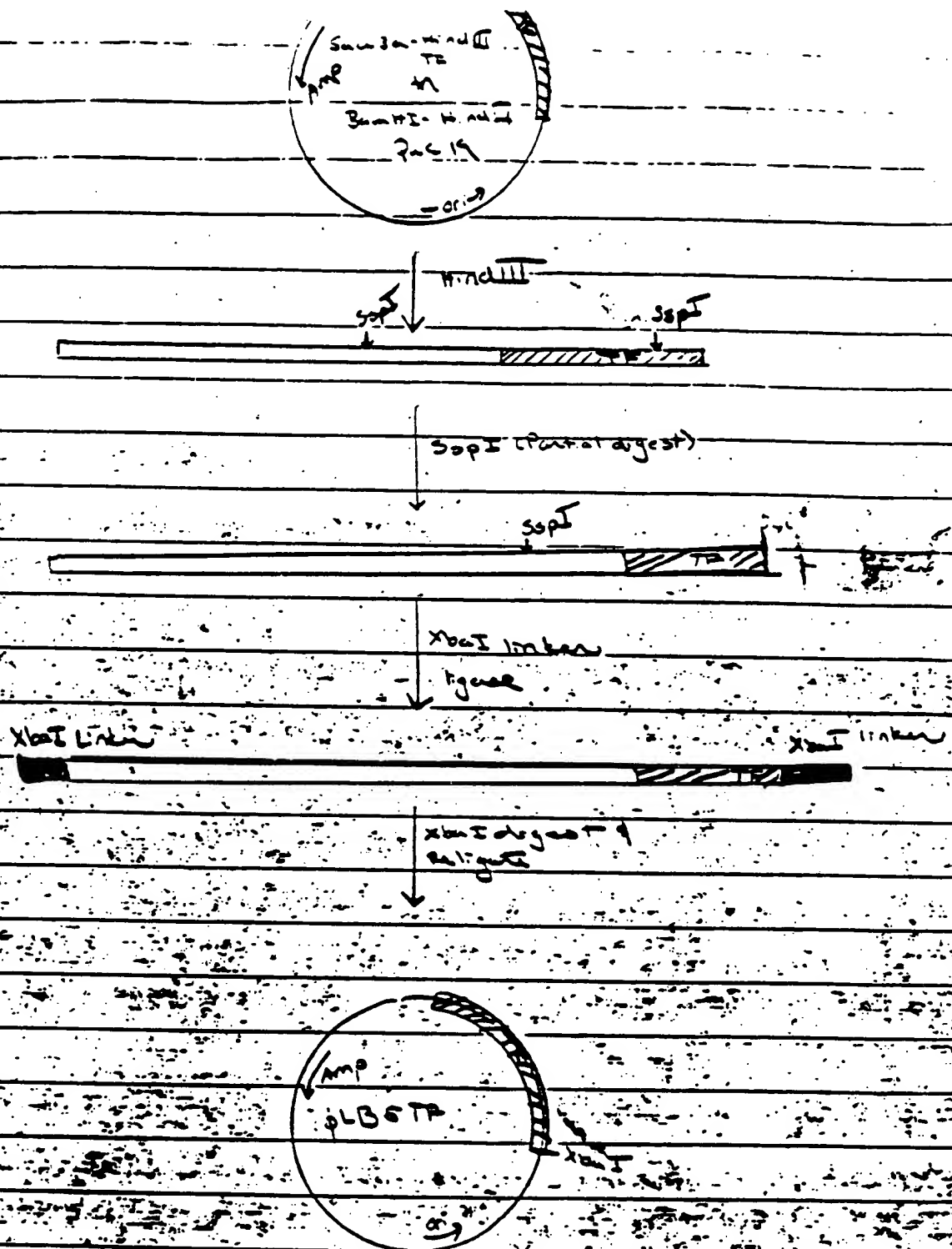


Fig. 10

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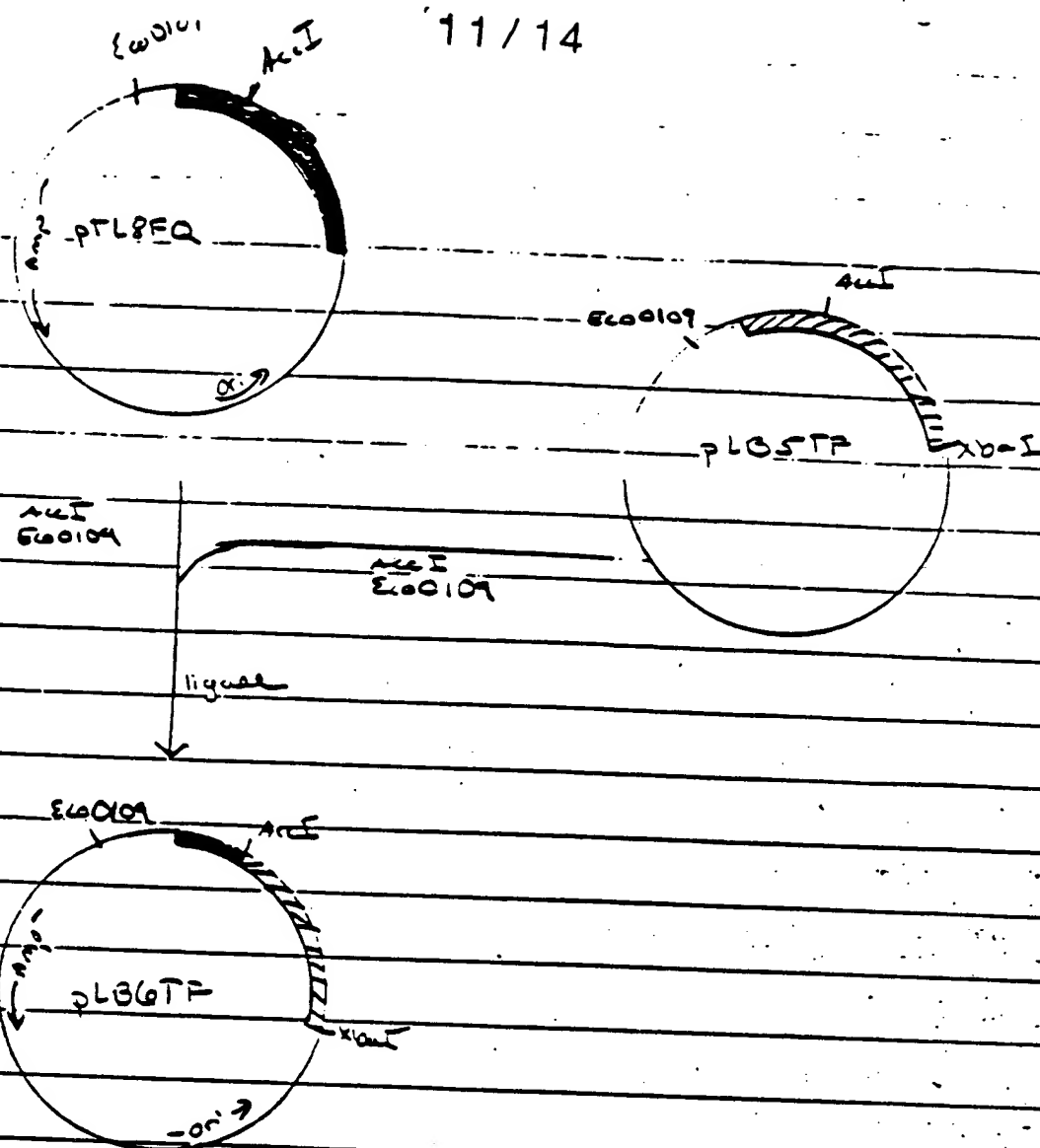


Fig. 11

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Fig. 12A

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Fig. 12B

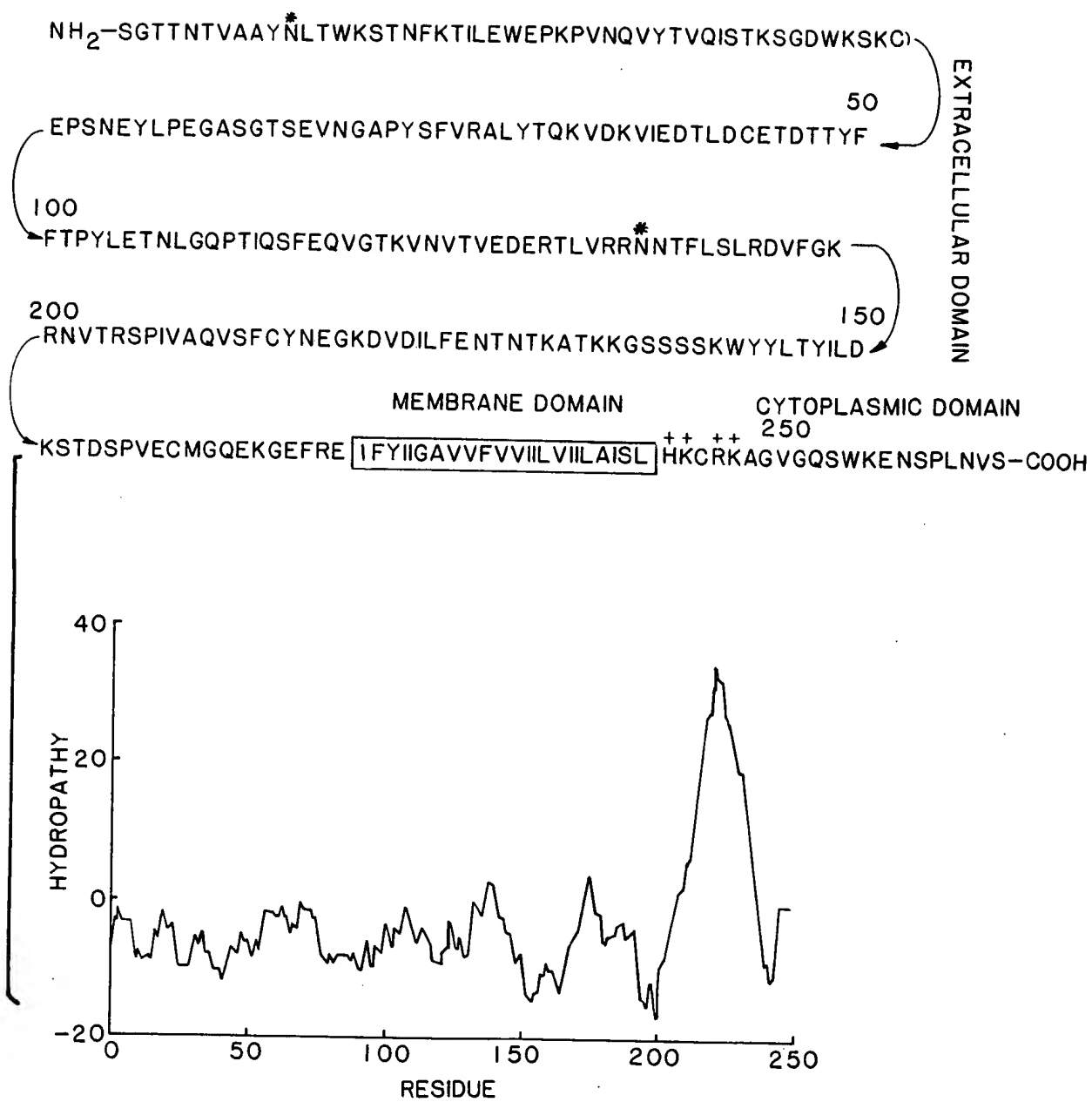


FIG. 1

FIG. 2

protein sequence (24-29)	Glu-Trp-Glu-Pro-Lys-Pro		
coding sequence	5'-GA _C ^A -TGG-GA _C ^A -CCX-AA _C ^A -CCX		
<u>Probe #1</u>	3'-CT _C ^T -ACC-CT _C ^T -GGX-TT _C ^T -GG-5'	mixture of 32	
<hr/>			
protein sequence (#145-159)	Asp-Val-Phe-Gly-Lys-Asp-Leu-Ile-Tyr-Thr-Leu-Tyr-Tyr-(Arg/Tyr)-Lys		
coding sequence	5'-GA _C ^T -GTX-TT _C ^T -GGX-AA _C ^A -GA _C ^T - ^A ₁ TX-AT _C ^T -TA _C ^T -ACX-CTX-TA _C ^T -TA _C ^T -AG _C ^A -AA _C ^A -3'		
<u>Probe #2</u>	3'-CTA-CAG-AAA-CCG-TTC-CTG-GAC-TAG-ATG-TGG-GAC-ATG-ATG-GCC-CTT-5'		
TF gene	5'-GAT-GTT-TTT-GGC-AAG-GAC-TTA-ATT-TAT-ACA-CTT-TAT-TAT-TGG-AAA-3' ★		

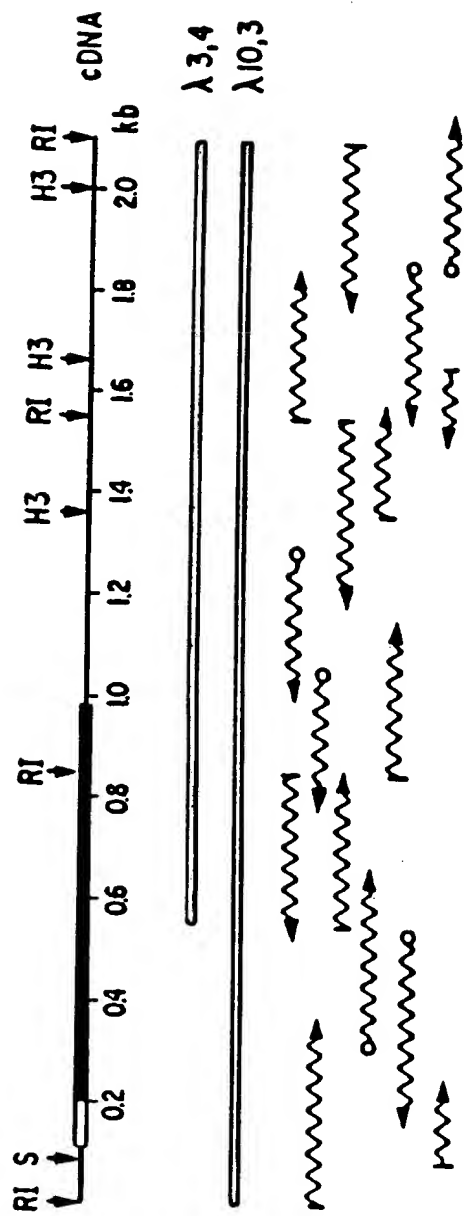


FIG. 3

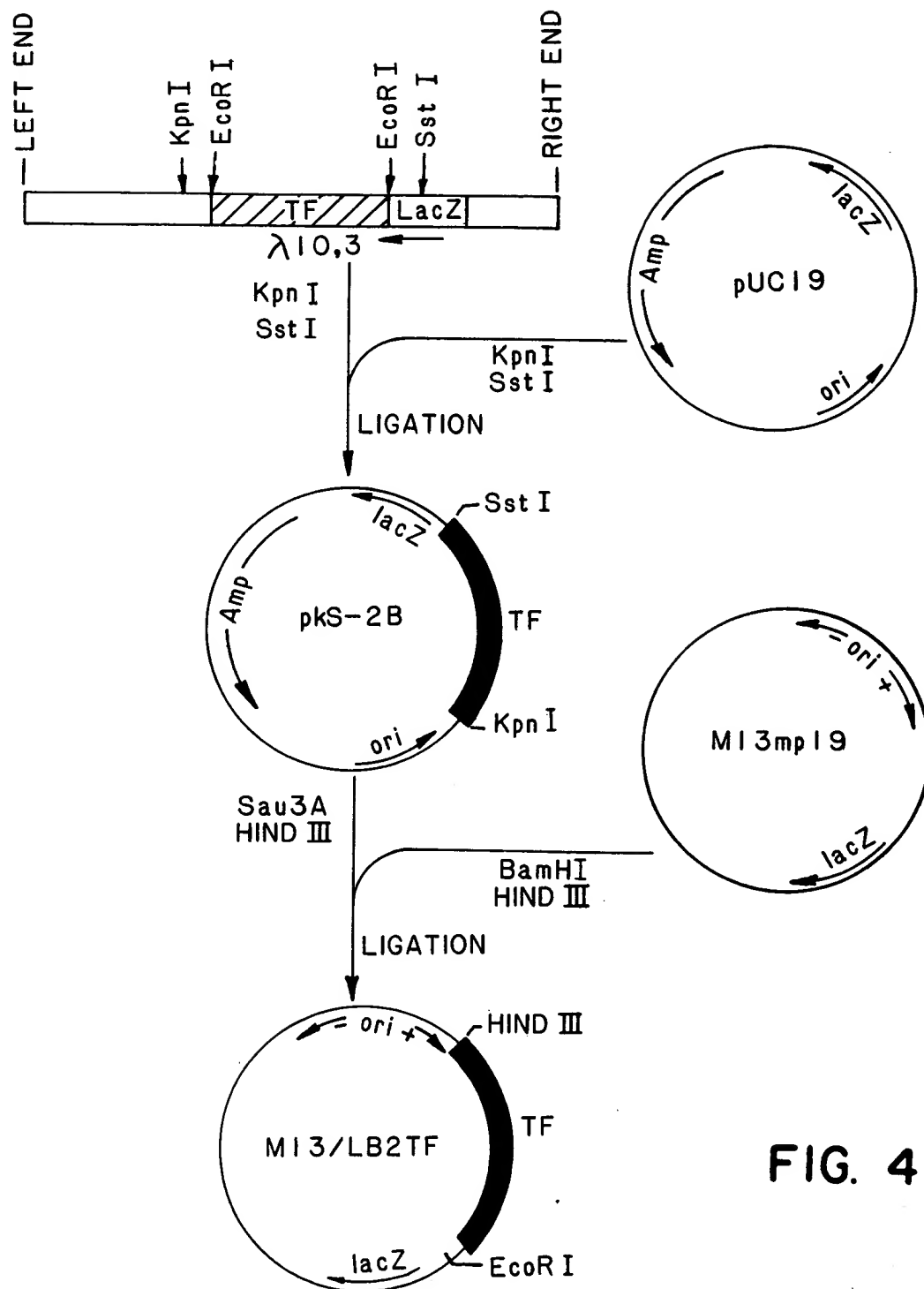


FIG. 4

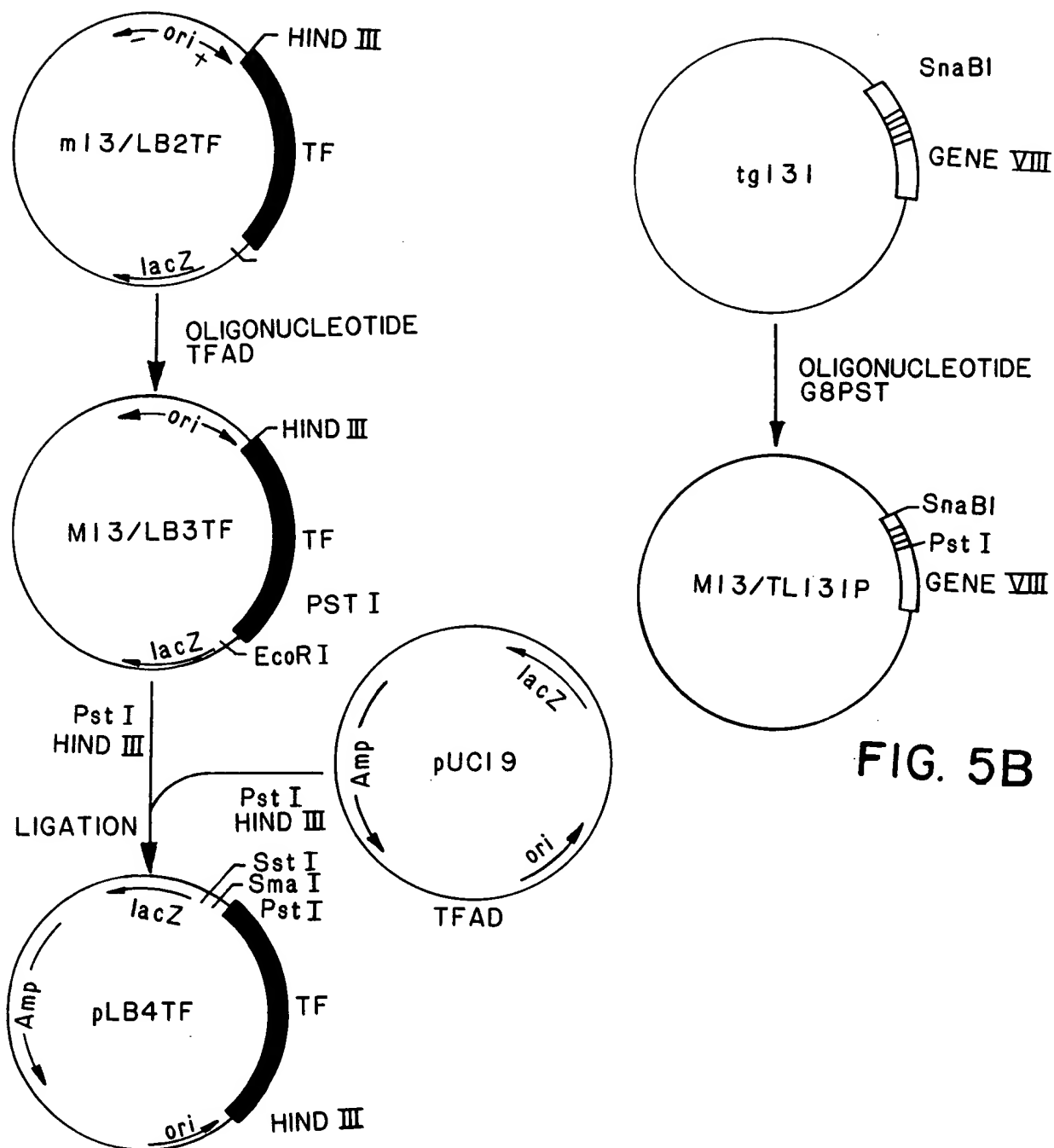


FIG. 5A

FIG. 5B

5' GGTGGCCGGCGCTGCAGATTCAGGCACTACA 3'
 G8PST
 5' GATCGTCACCCTCTGCAGCGAAAGAC 3'

FIG. 5C

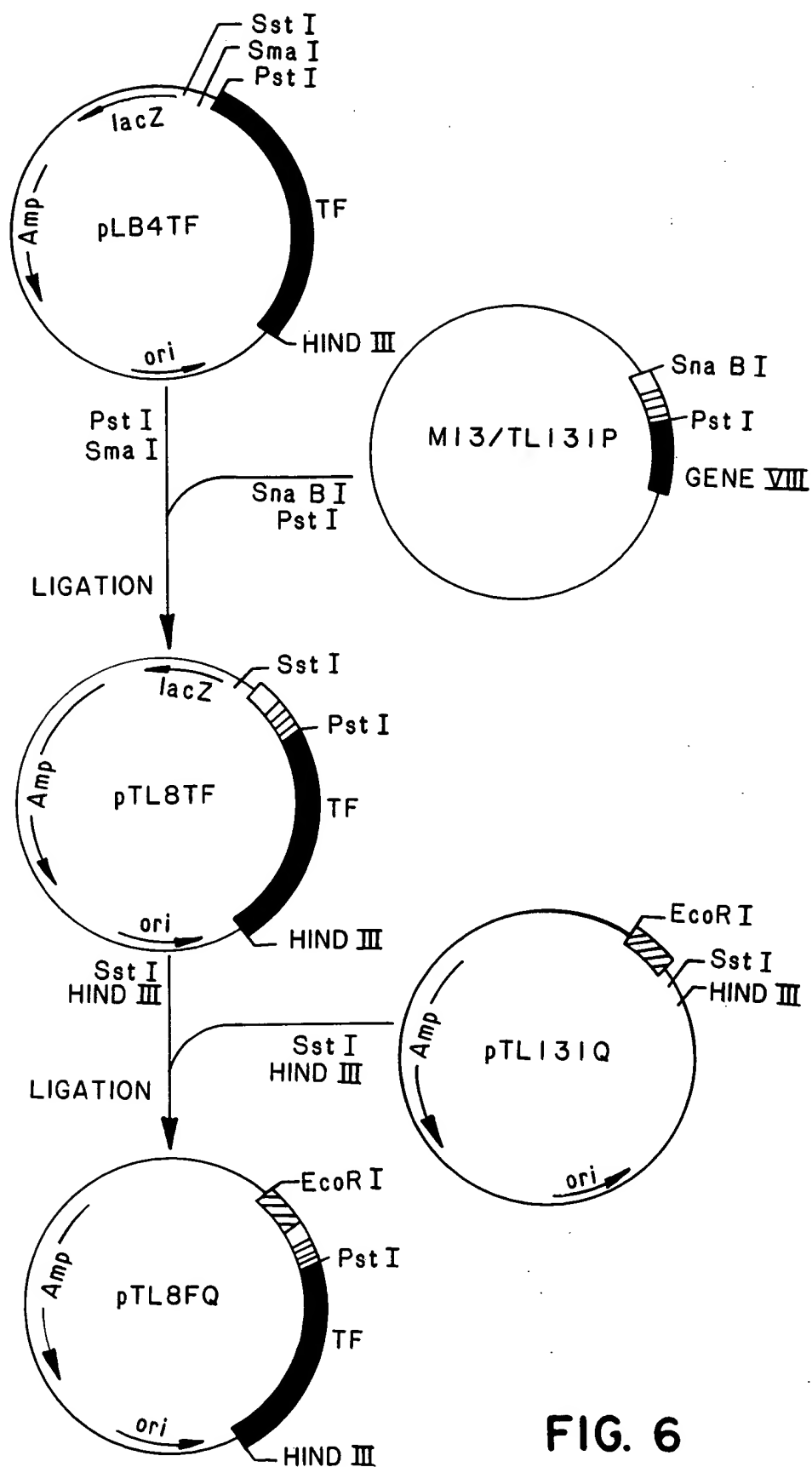


FIG. 6

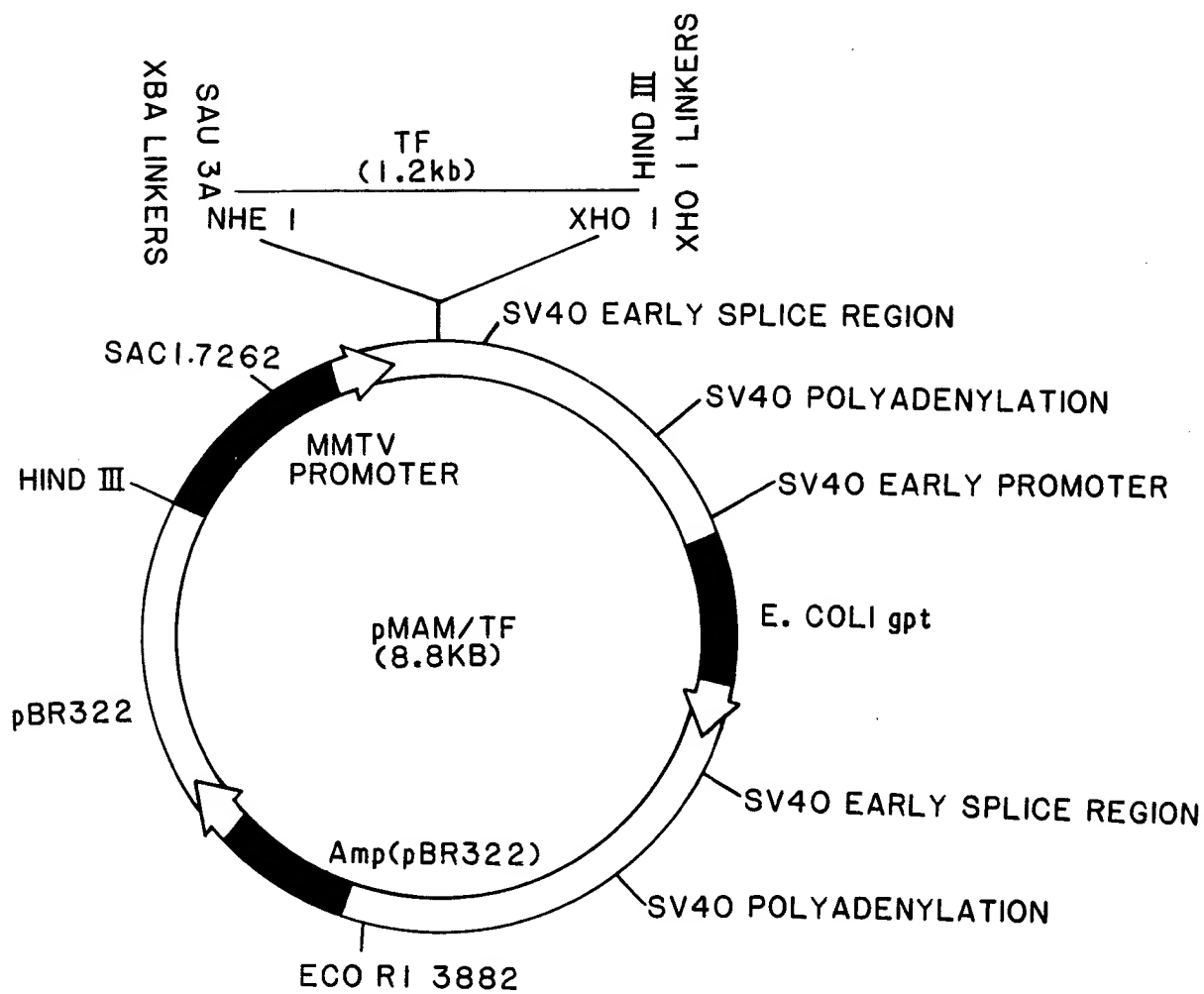


FIG. 7

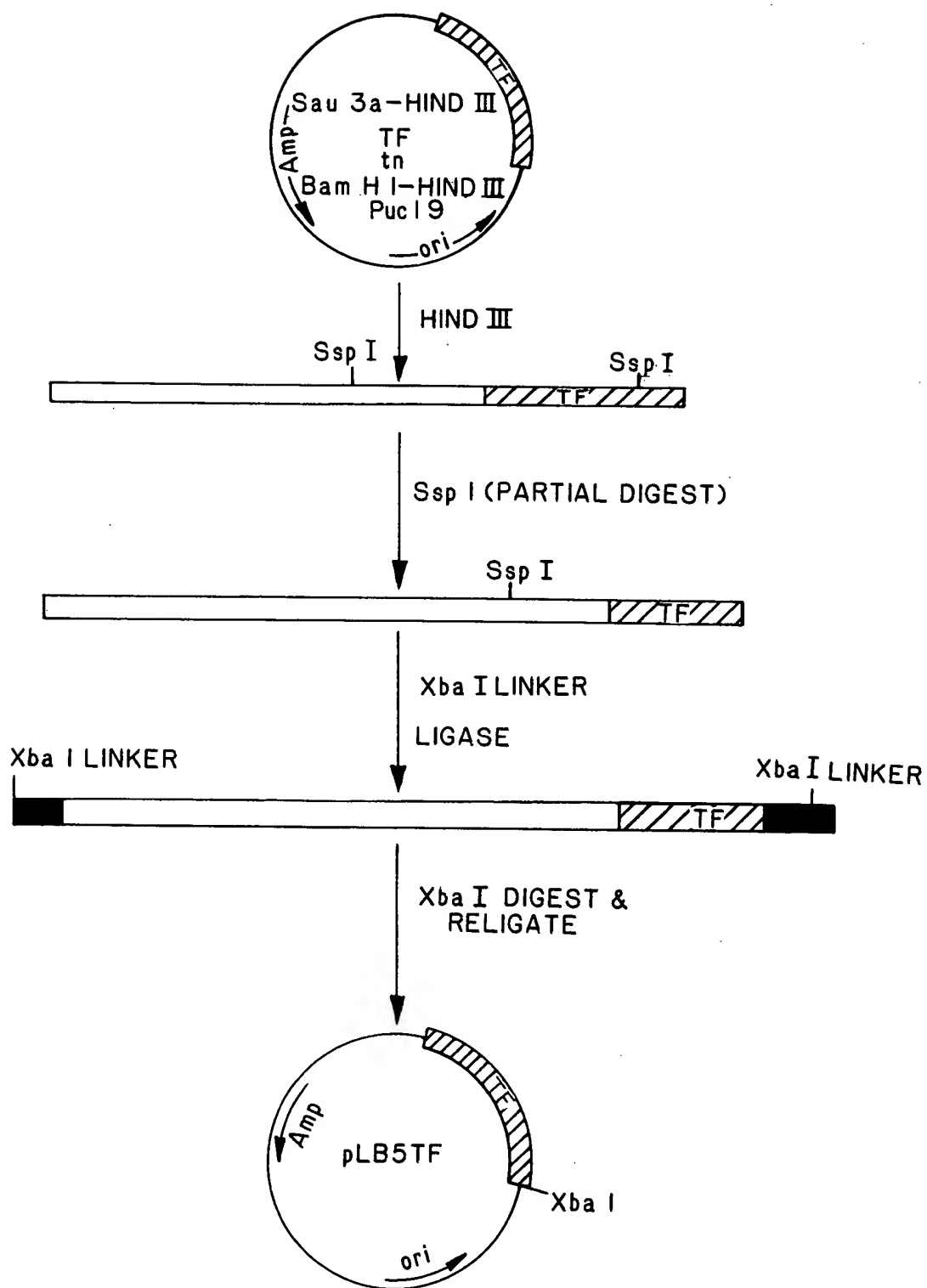


FIG. 8

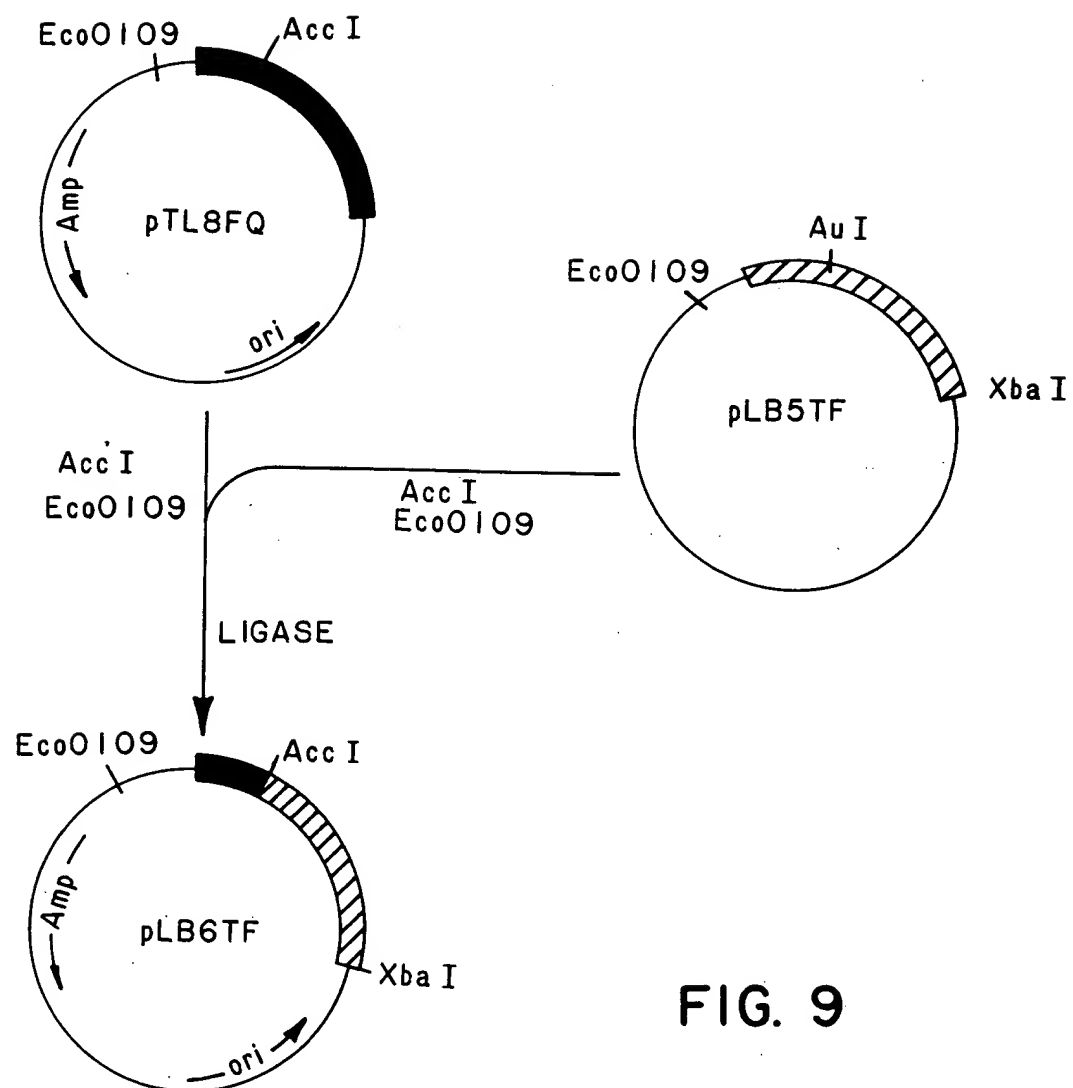


FIG. 9

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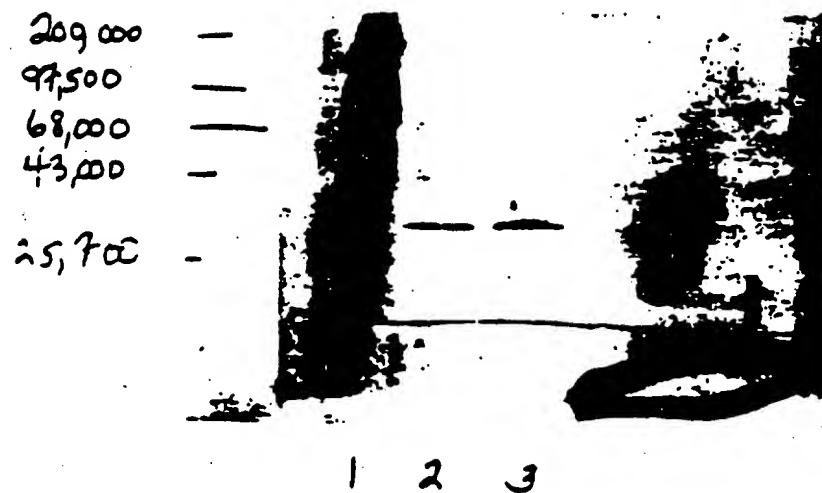


Fig. 12B

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